

**Optimization of Glucose Oxidase production and excretion
by recombinant *Aspergillus niger***

**Optimierung der Produktion und Exkretion von Glucoseoxidase
mittels rekombinanten *Aspergillus niger***

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This Work Is Dedicated To

The Memory of My Mother

My Father

and

All my Teachers From KG1 until Now

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1. Introduction

The success of *Aspergillus niger* for industrial production of biotechnological products is largely due to the metabolic versatility of this strain. *A. niger* is well known to produce a lot of organic acids, enzymes, plant growth regulators, mycotoxins and antibiotics. The industrial importance of *A. niger* are not limited on its more than 35 native products but also on the development and commercialization of the new products which are derived by modern molecular biology techniques

During the past few years numerous studies have been presented on *A. niger*, presumably the most important fungi for production and secretion of protein. The employment of *A. niger* as a host organism for production and secretion of homologous and heterologous proteins demonstrates many advantages such as:

- *A. niger* is a prodigious exporter species of homologous proteins and is able to produce certain enzymes in quantities of kilograms per cubic meter under the right conditions.
- *A. niger* has a long history of usage within the fermentation industry and is generally regarded as safe (GRAS). This often facilitates the path toward regulatory approval of the production system.
- The fermentation industries are very familiar with the conditions required to maximize production of homologous proteins in *Aspergillus*. Thus it provides a good starting point for the identification of physicochemical influences that are likely to be of greatest importance to heterologous protein production and secretion using a similar strain.
- *Aspergillus* is capable of carrying out efficient post translational modifications of products, e.g. glycosylation. This is especially important for some proteins derived from higher eucaryotes.
- *Aspergillus* species are effective secretors of proteins, often in an native, correctly folded form. They tend not to accumulate large quantities of the protein intracellularly, in form of inclusion bodies, as some bacteria and yeast do.
- *Aspergillus* has a useful production system for heterologous proteins derived from other filamentous fungi.

- Transformation stability is relatively high, therefore the threat of revertants is less pronounced.

Conversely a number of points can be made against *Aspergillus* as a potential system for proteins production which include:

- Low frequency of transformation in comparison with bacteria and yeast.
- The capability of *Aspergillus* to form a complex morphological structure, mycelial and pellet structures, which are usually associated with respect to mass and heat transfer limitation, mixing and controlling/monitoring the process as the concentration of the biomass increases.
- *Aspergillus* secretes significant quantities of other products such as organic acids which may reduce the pH of medium. This represents a diversion of the carbon energy source from the desired activity. A second possible difficulty lies in the effect on the protein being secreted into a low-pH environment. This may cause modification or even denaturation of the protein.
- *Aspergillus* is also capable of producing extracellular proteases. Such proteases could not only damage the product directly but also contaminate the final products and require an additional purification step.
- The production of fungal toxins should be also considered on using *Aspergillus* strains.

Glucose oxidase (GOx, β -D-glucose: oxygen, 1-oxidoreductase, EC 1.1.3.4) is a flavo-protein which catalyses the oxidation of β -D-glucose to glucono- δ -lactone with the concomitant reduction of molecular oxygen to hydrogen peroxide. GOx is used in food industries as a safe, natural, non-toxic preservative (to remove glucose and oxygen from food and beverages). In addition, GOx is also one of the most important enzymes in clinical analysis and in biosensor technology.

For many decades GOx was produced mainly as cell-associated enzyme by wild type *A. niger* requiring high concentration of oxygen and glucose as C-source. Immediately after the enzyme is produced, glucose is converted to gluconic acid which is a less favorable substrate for cell growth and GOx production.

Aim of this work

The aim of this work was to improve the process of glucose oxidase (GOx) production and excretion using a recombinant *Aspergillus niger* NRRL 3 (GOD 3-18) strain carrying multiple copies of the *god* gene fused to the α -amylase signal sequence and controlled by the *gpdA* promoter. This recombinant strain is able to produce the enzyme with higher amount and excrete it to the cultivation medium. To improve this process, cultivations were carried out in three different levels: Shake flask, 2 l bioreactor and 5 l bioreactor.

This work was focused on the following main tasks:

- (1) Improvement of GOx production on glucose by manipulation of growth morphology.
- (2) Change in medium composition and GOx induction with non-glucose C-sources.
- (3) Improvement of cell morphology to the desired form (growth in a small aggregate form, minimizing the mass transfer limitation inside the microbial pellet) with the aim to increase the GOx productivity. Change of cell morphology was done by cultivation under different hydrodynamic stresses conditions and with addition of a soluble biopolymer (xylan).
- (4) Improvement of GOx production process through fed-batch cultivation and production of GOx in high cell density culture.

Furthermore, a characterization of cell morphology during different stages (micro- and macro-levels) of cultivation with the help of image analyzer and biological staining method (to discriminate between the growing part and non-growing part of fungal pellet) was also desired. Using this method, a quantitative relation between growth form and productivity could be developed with the calculation of the productive and non-productive biomass within the growth population.

2. Review of Literature

2.1. Protein production and excretion by filamentous fungi

The filamentous fungi, typically, are saprophytic microorganisms which secrete a wide array of enzymes involved in the breakdown and recycling of complex biopolymers from both plant and animal tissues. Although the majority of these enzymes are hydrolytic and play an important role in fungal nutrition, releasing carbon and nitrogen locked in insoluble macromolecules obtained from the metabolic activities of other organisms. This makes the filamentous fungi as hosts for the production of secreted heterologous proteins (Jeenes *et al.*, 1991). Nowadays, the production of enzymes is an important and well growing sector of the fermentation industries. Several of these enzymes have been developed for a variety of commercial uses; for example in textile processing, leather manufacturing, paper and pulp processing, detergent production, and in food processing. Some fungal strains which are used to produce enzymes in an industrial process are capable to secrete large amounts of the respective products. For example, *Aspergillus niger* produces glucoamylase at 0.5 g l^{-1} , as a result of mutation as well as medium development and optimization of fermentation condition, the yield increased 40 folds reaching about 20 g l^{-1} (Finkelstein, 1987). *Trichoderma reesei* produces cellulases at 30 g l^{-1} , the major component of the complex cellulases is cellobiohydrolase I. All of these cellulases are heat stable. However, they produce cellobiose as an end product which has feed back inhibition on the enzyme activity. *T. reesei* cellulase productivity was improved more than four fold by strain improvement programs (Durand *et al.*, 1988). The capacity of filamentous fungi for high level protein secretion was one of the key features in considering them as potential host for producing high value recombinant therapeutic proteins (Davies, 1991). One strategy to improve the production of heterologous protein in recombinant microorganisms is the development of secretion systems (Nicaud *et al.*, 1986). Export of the produced protein from the host cell reduces the risk of protein degradation by intracellular proteases, allows glycosylation and protein folding to occur, simplifies down-stream purification, and reduces the effect of feed back inhibition mechanisms if present in the production pathway. Product secretion is also desired if the protein is toxic to the host cell (Wang and Da Silva, 1993). Moreover, organisms such as *A. niger* and *A. oryzae* have a long history of usage within the fermentation industries and are generally regarded as safe (GRAS) in accordance with the food and drug administration (FDA). Therefore, the development of an expression system in these

microorganisms is desired. Also, the production of chymosin using *A. niger* var *awamori* was extensively studied by Dunn-Coleman *et al.* (1991). Bovine chymosin production increased up to 1 g l⁻¹ after gene expression in *A. niger*.

2.1.1 Hyphal growth and protein secretion

The relation between the cytology of hyphal growth and protein excretion in filamentous fungi was observed since a long time ago. The protein excretion by filamentous fungi is probably restricted to the tips of growing hyphae. The hyphal tips were shown to be free of all organelles, except for a large number of vesicles of varying size, and some of them were seemed to be in the process of fusing with the plasma membrane (Peberdy, 1994). This observation led to the idea that the vesicles were involved in the transport of materials to the surface of the plasma membrane at the hyphal tip, as well as in membrane growth. Using immuno-cytochemical methods, it was shown that glucoamylase secretion in *A. niger* occurred predominantly at the growing hyphal tip (Wösten *et al.*, 1991). On the other hand, the secretion of enzymes involved in lignin degradation by *Phanerochaete chrysosporium*, a process associated with the non-growing phase in the physiological cycle of fungal culture, was also shown to be associated with the tips of newly formed hyphal branches. Generally, in other eukaryotic cells, it is presumed that the apical vesicles are the final step of the intracellular secondary pathway that begins at the endoplasmic reticulum (ER) and proceeds via a Golgi system. To date, a Golgi system has only been described in Oomycetes. However, other types of fungi are thought to have organelles that are equivalent to the Golgi structure (Peberdy, 1994).

The available evidence clearly points to protein secretion being a high polarized process involving the movement of protein containing vesicles to the hyphal tip. The secretory vesicles appear to be associated with microtubules and, it is probable, move along with them via an ATP dependent process. A hypothetical secretory pathway in the hyphae of filamentous fungi is shown in **Fig. 2.1**. In eukaryotic cells, the desired proteins for secretion are synthesized on ribosomes of the endoplasmic reticulum. The secretory process is then initiated by the sequencation of the nascent extracellular portion into the lumen of the rough endoplasmic reticulum (RER). This process is determined by the information of the signal sequence attached to the protein molecule. In general, signal sequence of different organisms share the common feature. They comprise 13-30 amino acids with a basic N-terminal region and more polar C-terminal region, which is the cleavage site.

The hypothesis implicating protein-conducting channels in the ER membrane was described by High (1992).

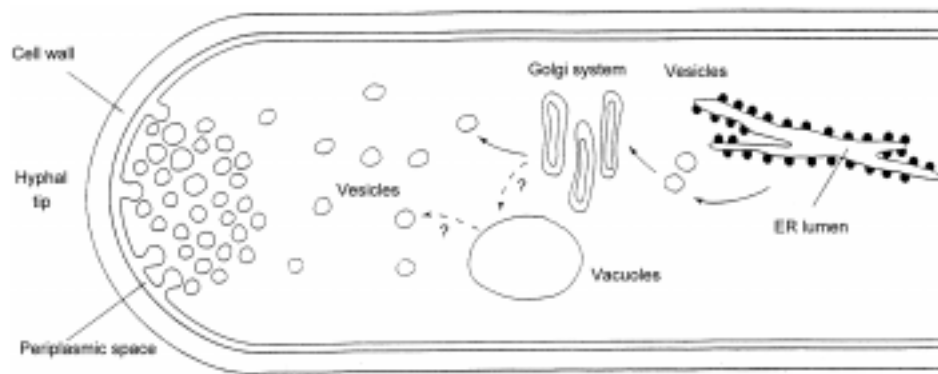


Figure 2.1: A hypothetical secretory pathway in filamentous fungi (Peberdy, 1994).

Hence, the protein is transported through the organelles of the secretory pathway, the signal peptide sequence is cleaved from the nascent protein by an endopeptidase contained within the ER lumen [Fig. 2.2].

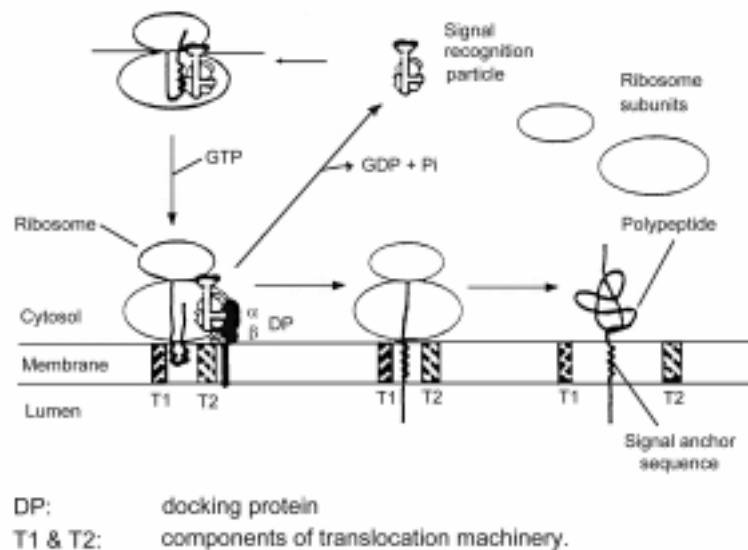


Figure 2.2: Targeting of a secreted protein to the endoplasmic reticulum membrane (High, 1992).

Before proteins are secreted, they undergo several post-translational modifications. This starts in the ER and continues as the protein pass through the secretory vesicles. Three changes may occur to a protein molecule:

- 1- Proteolytic cleavage to remove the signal sequence and other peptide sequence, if present.
- 2- A folding process involving the formation of disulphide bonds to develop the tertiary and quaternary structure of the protein, where the disulphide bonds stabilize the molecule.
- 3- Glycosylation.

These maturation processes involve several different enzymes which are present in the ER.

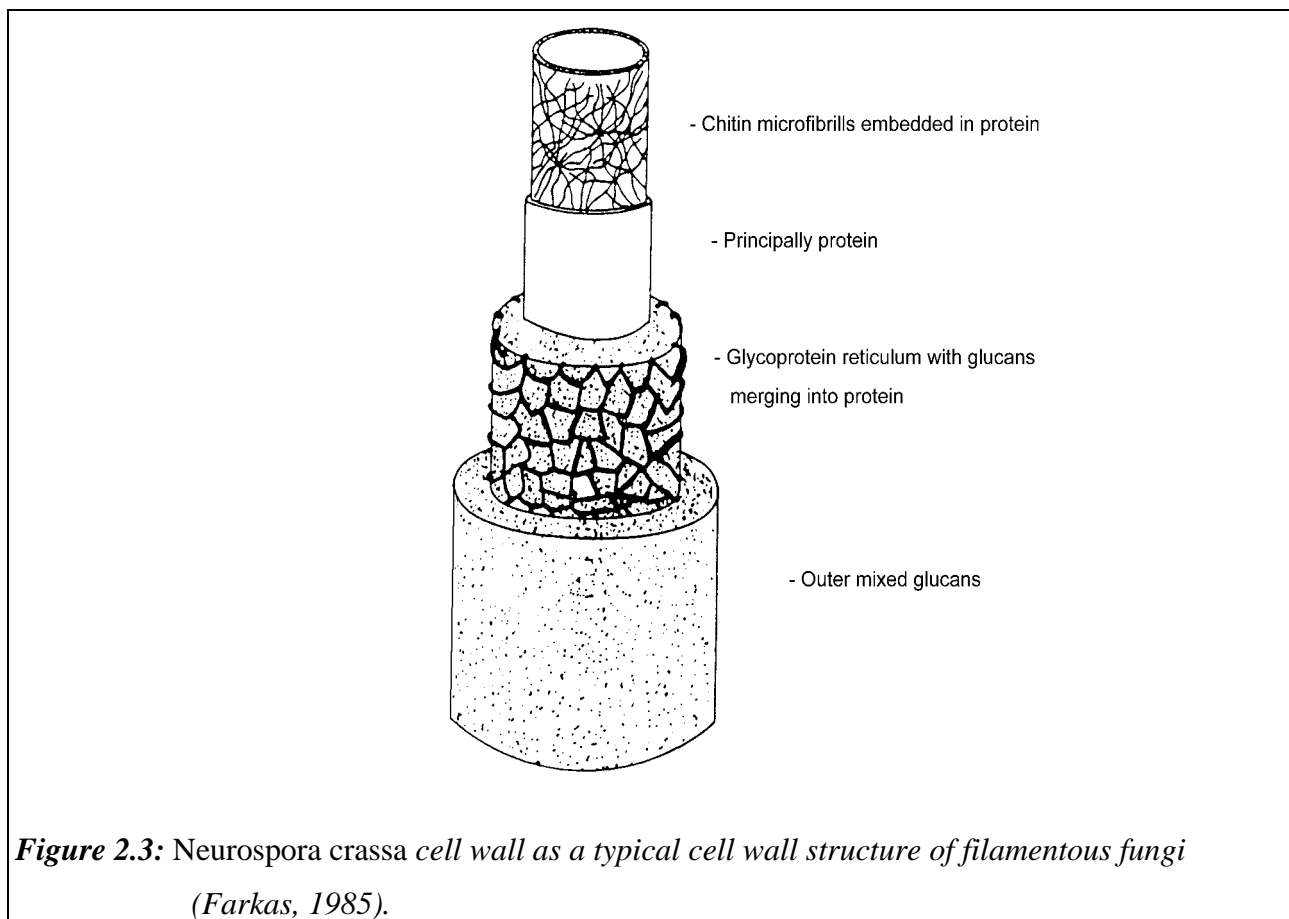
2.1.2 Cell wall and protein excretion

The fungal cell wall fulfills several functions connected with the interaction between the cell and their environment. Some of these are:

- 1- Formation of a rigid, mechanical barrier on the surface of the protoplast to determine the cell shape.
- 2- Protection from osmotic stress on the protoplast.
- 3- Acting as a carrier of specific antigens characteristics of the particular cell and playing an important role in cell recognition in various cell interactions.
- 4- Acting as the site of various extracellular enzymes engaged in the exchange of nutrients and/or products of metabolism as well as hydrolysis of cell wall components during the cell growth.
- 5- Acting as a reservoir of carbohydrates, which can be reutilized under limiting conditions or in certain stage of the life cycle.

The chemical composition of the cell wall is closely correlated with the taxonomic classification of fungi. In general, the fungal cell wall is shared in common chemical structure composed of homo- and heteropolysaccharides, protein, protein-polysaccharide complex, lipid, melanin and polysaccharide chain of chitin. The unique mechanical, chemical and biological properties of fungal cell wall is not only in their chemical composition but also in the mode of spatial arrangement of the individual polymers. The layering of the cell wall components is one of the most characteristic ultrastructure of fungal cell walls. The general picture is that the skeletal, microfibrillar wall components, such as β -glucan, chitin, and/or cellulose, are embedded in an amorphous polysaccharide and protein-polysaccharide matrix. The outer surface of the wall is

usually smooth or slightly rough, whereas the skeletal polysaccharide microfibrils are more prominent on the inner surface of the wall (Farkas, 1985). As an example, the cell wall structure of the hyphal cell wall of *Neurospora crassa* is described in **Fig. 2.3**. The hyphal walls of *N. crassa* consist of coaxial layers of individual wall components and the chitin microfibrils in the innermost wall layer are covered by proteinaceous material and glycoprotein reticulum. The outermost wall layer is smooth, composed of mixed α - and β -glucans (Burnett, 1979). Until now, there is only a little information available about the nature of linkage between the different components in the fungal walls. The existence of covalent linkages between chitin and glucan has been described in *A. niger* (Stagg and Feather, 1973).



In eukaryotic cells, the cell wall plays an important role as a biobarrier for nutrient uptake and excretion processes. In the process of excretion, the determination of the molecular threshold of cell walls suggests that the size limit is around 20,000 k Dalton (Peberdy, 1994). The location of enzyme after the release from the surface of the cytoplasmic membrane is not clearly defined and

the excretion processes are highly dependent on the porosity of the cell wall. In wild type *N. crassa*, invertase remains in the periplasmic space whereas in the mutant form it is excreted outside the cell due to the increased porosity of the cell wall (Trevithick and Metznerberg, 1966). As the porous and nascent apical wall of fungi is transformed to the less porous lateral wall during growth, some exoenzymes are trapped and become bound within the cell wall. The hypothesis conflicting excretion and retention of exoenzyme by the wall is based on the structural and physiological differences between the apical and lateral wall of hyphal fungi as described by Chang and Trevithick (1974). Proceeding to the apical region within 2 μm at the hyphal tip are zones called (α , β , γ) as shown in **Fig. 2.4**.

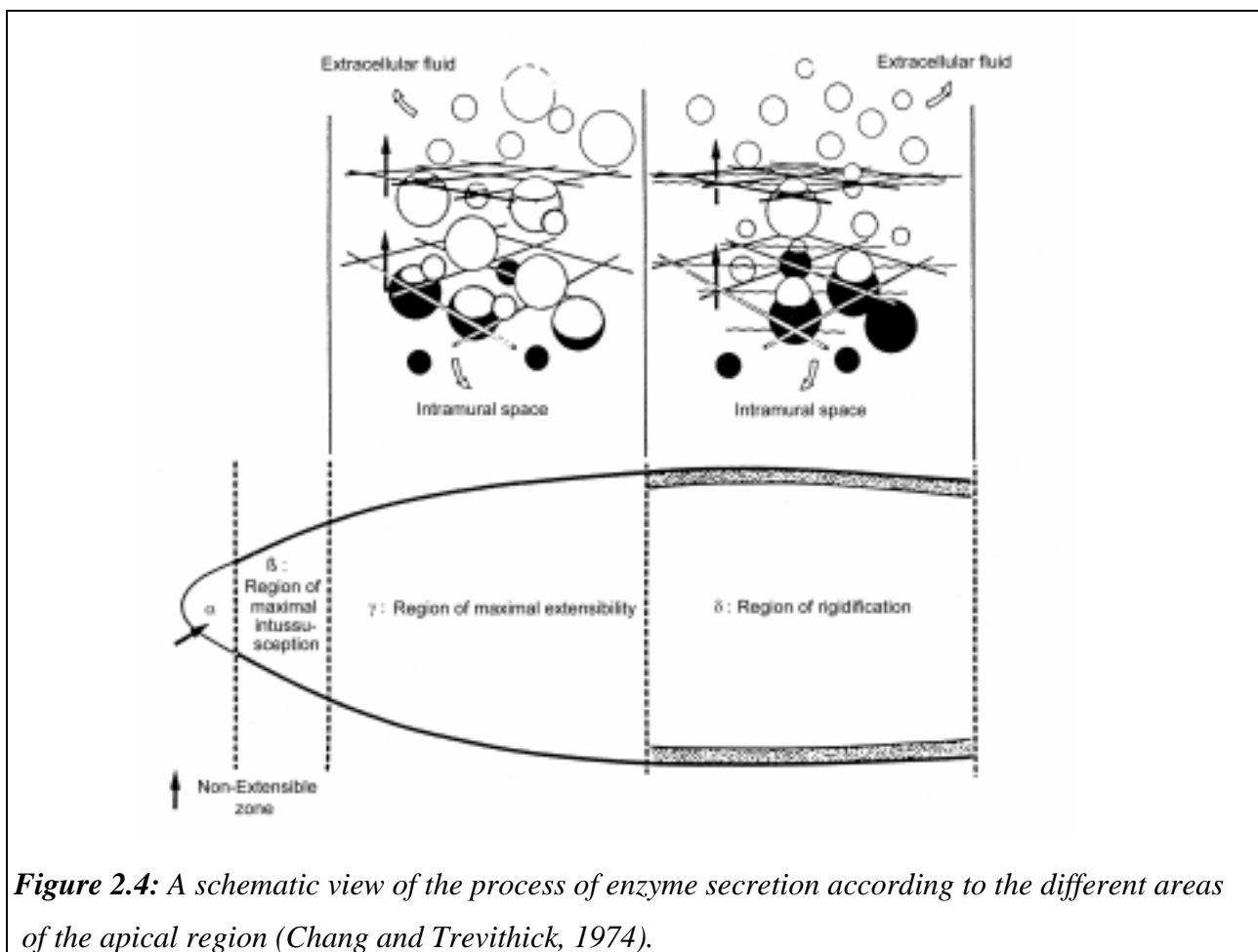


Figure 2.4: A schematic view of the process of enzyme secretion according to the different areas of the apical region (Chang and Trevithick, 1974).

Of particular interest are the β zone of maximum intussusception of new wall material and the highly elastic and extensible γ zone. Both of these zones are mechanically weak. The rest of the lateral hyphal wall, the δ zone is rigidified by secondary wall substances. The transformation of

the apical into the lateral region may be responsible for the fraction of exoenzymes retained in the walls. During the process of rigidification of the pores in the wall (δ zone) which are initially large enough to release the macromolecules from the intramural or periplasmic space. The pores of this zone become smaller due to the addition of secondary wall material (Chang and Trevithick, 1974). Consequently, this portion of exoenzyme becomes trapped during the transit and corresponds to the wall bound fraction. An alternative hypothesis is offered by the „bulk-flow“ hypothesis, which assumes that proteins excreted by the very tip are pushed through the wall to the outside of the wall by accretion of plastic wall polymers during apical wall growth (Wessels, 1990). Therefore, the factors which increase the extent of hyphal branching may improve the yield of enzyme secretion in filamentous microorganisms (MacKenzie *et al.*, 1994).

The external/internal ratio of enzyme was found to be strain dependent in accordance with the differentiation in cell wall composition. For example, invertase is excreted by both *A. niger* and *A. nidulans*. In *A. niger*, the distribution of the enzyme is 70% cell bound and 30% excreted, but in *A. nidulans*, the enzyme is distributed more equally. In all cases, 70% of the cell bound enzymes are external to the plasma membrane (Peberdy, 1994). If the cell wall is impaired, by using mutant strain such as mutant *N. crassa* (Bigger *et al.*, 1972), or removed, as in the protoplast of *A. nidulans*, the level of excretion of invertase reaches around 90%. Moreover, the cell wall rigidity could be controlled by the inhibition of chitin synthesis, the most important skeletal structure of fungal cell wall, through the depletion of divalent cations in the cultivation medium. Among different divalent cations tested, Ca^{2+} and Co^{2+} increase the activity of chitin synthetase in *Phycomyces blakesleeanus* whereas Mg^{2+} , which is the most efficient divalent cation in the stimulation of enzyme catalysis, proved ineffective in the activation process (Martinez-Cadena and Ruiz-Herrera, 1987).

2.1.3 Cell morphology and protein excretion

2.1.3.1 Fungal cell morphology

The morphological growth forms of filamentous organisms in the aerobic submerged cultivation may lead to suspension characteristics quite different from bacterial and yeast cultures. The macro-morphological features of the filamentous microorganisms, which have a significant effect on the rheological properties of the cultivation medium, reflected directly in the production and excretion of different microbial metabolites. In submerged cultivation involving filamentous organisms, the morphology can vary from discrete compact pellets of hyphae to homogeneous suspension of dispersed mycelia. These morphological differences are associated with significant differences in growth kinetics and physiology. Growth of dispersed mycelia is effectively equivalent to that of unicellular, with homogeneous distribution of biomass, substrate, and products and exponential growth at a constant specific rate in batch culture where substrates are in excess.

The filamentous form of mycelial hyphae easily causes entanglement, and the cultivation broth becomes very viscous. The rheological behavior is usually non-Newtonian, leading to relatively low viscosities in regions of high shear rate (near the impeller) and very high viscosities in region with low shear rate (near the wall). The high viscosity and pseudo-plasticity of the suspension cause many problems during the cultivation, decreasing the mass transfer, heat transfer and requiring more power input for mixing. In this case, only a small part of the bioreactor, around the impeller, is maintained at the optimal condition. Increasing the agitation rate improves the overall homogeneity, but this also rises the power consumption and often damages the cells due to the high shearing (van Suijdam and Metz, 1981; Braun and Vecht-Lifshitz, 1991). On the other hand, the pellet form, in the macroscopic feature, can be an attractive growth form for cultivation of fungi. The most important advantages are the decrease of the viscosity of the cultivation broth and the rheological properties become Newtonian. The Newtonian fluid is characterized by good mass and heat transfer properties. Moreover, the pellet formation facilitates the separation of fluid in down stream processes (van Suijdam *et al.*, 1980). The macroscopic features of cultivation medium containing fungal pellets show homogeneity in their rheological properties. On the other hand, the microscopic scale of pellet shows some heterogeneity due to the zonation in accordance with the different hyphal densities

inside the pellet. The zonation process has been discussed by many authors (Kobayashi *et al.*, 1973; König and Schügerl, 1982; Lawton *et al.*, 1989; Buschulte *et al.*, 1991; Pottel and Bellgardt, 1992). As long as there is sufficient supply of oxygen to all cells within the pellet, it grows in density as well as in size. After some time, the oxygen concentration in the center of the pellet drops to almost zero, restricting cell growth to a zone next to the pellet surface. The limitation of oxygen supply and the removal of metabolic products can lead to an alteration of the cellular metabolism and enzyme excretion kinetics (Hermersdörfer *et al.*, 1987). In studies of penicillin production using *Penicillium chrysogenum* pellets, the transfer resistance inside the fungal pellet is high in large pellet. This causes an oxygen deficiency and autolysis of the cells at the center of the pellet. However, the thickness of the layer which contain the living cells remains constant, regardless of the pellet size. The resistance of the gas/liquid interface outside and inside the pellets are equal for pellets 400-500 μm in diameter (Schügerl *et al.*, 1983; Wittler *et al.*, 1986). Pellets smaller than 400 μm in diameter consists of a metabolically active layer only, and all the cells are supplied with sufficient oxygen.

In case of growth in pellet form, the microbial growth is affected by pellet morphology as discussed above, there being two extremes. In pellets consisting of densely packed hyphae, growth is restricted by diffusion of material from the liquid phase to the pellet center and unrestricted growth is limited to the hyphae in an outer peripheral shell. Thus, in batch culture, biomass (M) increases as a cubic function of time [Eq. 2.1].

$$M^{1/3} = kt + M_o^{1/3} \quad [2.1]$$

where M_o represents the initial biomass and k is a constant. If a culture is assumed to consist of n spherical pellets, of equal radius r and density ρ , with an active outer mycelial shell of width w , growing at a specific rate μ , then the constant k can be determined as follows:

$$k = \left(\frac{4}{3} \pi \rho n \right)^{1/3} \mu w \quad [2.2]$$

where k , represent the rate by which pellet radius increase due to growth. ρ , pellet density, n , constant or number of pellets and W , width of active outer mycelial shell.

When a pellet exceeds a certain size it is assumed that growth is limited to a peripheral zone of thickness w , through limitation of the rate of penetration of the growth-limiting nutrient.

Although cube-root growth kinetics have been observed in fungal culture, experimental data do not always allow distinction from other models. In addition, the cube-root law fails to consider the effects of mass transfer and substrate concentration on growth. Attempts have been made to consider oxygen uptake, consumption, and limitation within a pellet (van Suijdam, *et al.*, 1982) but most do not take into account the variation in pellet size, density, and micro-morphology which occur in liquid culture. Such variation is of significance because it is the pellet size frequency distribution which defines the mount of mycelium in contact with the growth medium, the proportion of biomass which is growing, and the rate of substrate utilization.

On the other hand, if the pellets consists of a loose, open, more filamentous mycelium, agitation of the cultures allows nutrients and oxygen to reach all constituent hyphae and supports exponential growth of the entire biomass. The latter type of growth is also more easily controlled because, in the ideal state, all of the hyphae are growing exponentially and all are in contact with well-stirred medium, so all can respond rapidly to manipulation of the medium. These advantages have to be traded off against increased viscosity caused by the filamentous growth. Thus the ability to control the morphology of a fungus in submerged culture is important, since morphology can affect production yield.

2.1.3.2 Fungal pellet classification

Based on the mechanism of pellet formation, pellet classification in three types: coagulative, non-coagulative and hyphal element agglomeration type, has been distinguished by Nielsen, (1996). The coagulative type is characterized by the coagulation of spores while germination gives rise to a net of intertwined hyphae. *A. niger* pellets were found to be a good example of this type. In non-coagulative type, one spore gives rise to one pellet and the number of pellets are directly correlated with the number of spores used as inoculum. Some species of *Streptomyces* belong to this group. In the hyphal element agglomerating type, the hyphal elements agglomerate and form a clump of hyphal elements which eventually evolve into pellet. *P. chrysogenum* has been shown to belong to this group. In case of enzyme production,

the macro-morphological feature of fungi is not only affecting the enzyme production and excretion but also has a significant effect on the reactivity.

2.1.3.3 Factors affecting microbial pellet formation

The process of microbial pellet formation and mycelial cell aggregation is influenced by many factors. These factors include strain used, growth rate, medium composition, addition of surfactants, polymer addition, shear force, aeration and agitation (e.g. Elmayergi *et al.*, 1973; Mitard and Riba, 1988; Hotop *et al.*, 1989; Smith *et al.*, 1990 and Nielsen *et al.*, 1995). Generally, these factors could be classified into three main categories, strain dependent factors, nutrition dependent factors (medium composition) and cultivation conditions as shown in **Fig. 2.5** and **Tab. 2.1**.

The interaction between these factors has to be considered and it is not easy to determine or discuss some of these factors separately. For example, the effect of cultivation vessel shows influence on the oxygen transfer inside the cultivation medium as well as on the shear force. Also, the effect of cultivation conditions such as: temperature, oxygen supply, etc. as well as medium components such as: C-source, N-source, C/N ratio, etc. will reflect in the specific growth rate of the microorganism. Moreover, the interactions between these factors should be also considered. For example: the effect of inoculum size, a strain dependent factor, has an effect on the aeration in the submerged culture of the fungal cells (Brown and Zainudeen, 1978).

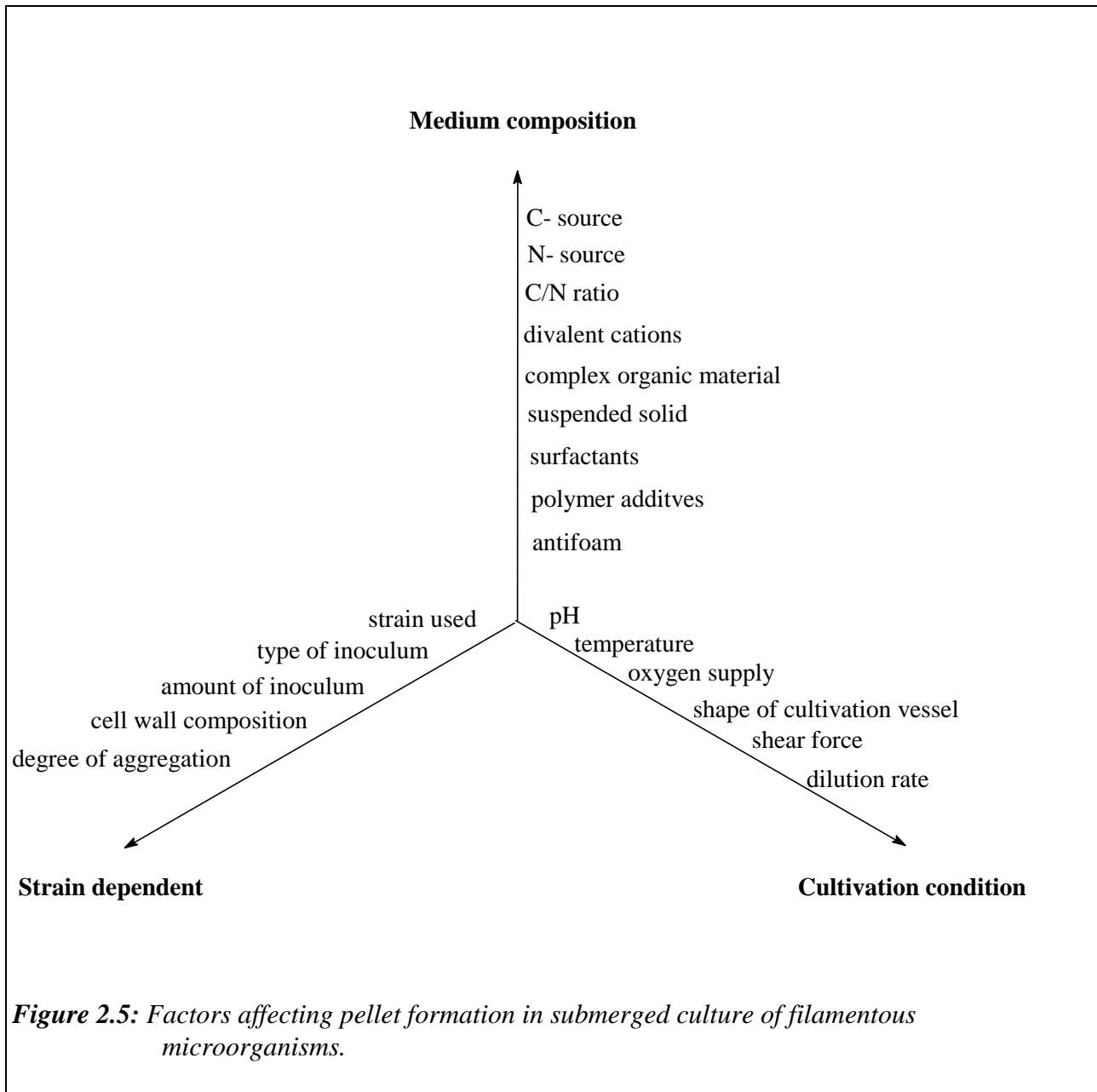


Table 2.1: Effect of different factors on the production of microbial pellets

Factor	Microorganism	Reference
Strain dependent factors		
- Strain used	<i>Actinomycetes</i>	Lawton <i>et al.</i> , 1989
- Amount of inoculum	<i>Penicillium chrysogenum</i>	Tucker and Thomas, 1992
- Type of inoculum	<i>Penicillium chrysogenum</i>	Hotop <i>et al.</i> , 1989
	<i>Aspergillus niger</i>	van Suijdam <i>et al.</i> , 1980
- Type of aggregate	<i>Aspergillus niger</i>	Olsvik <i>et al.</i> , 1993
- Cell wall composition	<i>Streptomyces tendae</i>	Vecht-Lifshitz <i>et al.</i> , 1990
- Growth rate	<i>Penicillium chrysogenum</i>	Morrison and Righelato, 1974
	<i>Penicillium chrysogenum</i>	Righelato <i>et al.</i> , 1968
	<i>Aspergillus nidulans</i>	Katz <i>et al.</i> , 1972
Nutritional dependent factors		
(medium composition)		
-Addition of complex organic material	<i>Rhizopus arrhizus</i>	Byrne and Ward, 1989
- Carbon source	<i>Aspergillus niger</i>	Hermersdörfer <i>et al.</i> , 1987
- Nitrogen source	<i>Aspergillus niger</i>	Hermersdörfer <i>et al.</i> , 1987
- C/N ratio	General idea	Braun and Vecht-Lifshitz, 1991
- Addition of polymer	<i>Aspergillus niger</i>	Elmayergi, 1975
	<i>Streptomyces coelicolor</i>	Hobbs <i>et al.</i> , 1989
	<i>Streptomyces lividans</i>	Hobbs <i>et al.</i> , 1989
	<i>Penicillium chrysogenum</i>	Pedersen <i>et al.</i> , 1993
	<i>Mortierella vinacea</i>	Kobayashi and Suzuki, 1972
- Addition of surfactants	<i>Aspergillus niger</i>	Elmayergi <i>et al.</i> , 1973
	<i>Aspergillus niger</i>	Metz and Kossen, 1977
-Addition of divalent cations	General idea	Clark and Lentz, 1963
-Presence of solid particles	<i>Aspergillus niger</i>	Metz and Kossen, 1977

Table 2.1. cont.

Factor	Microorganism	Reference
Cultivation conditions		
- pH	General idea	Braun and Vecht-Lifshitz, 1991
- Temperature	<i>Aspergillus niger</i>	Hermerdörfer <i>et al.</i> , 1987
- Oxygen supply	General idea	Olsvik and Kristiansen, 1992
	<i>Aspergillus niger</i>	Zetelaki and Vas, 1968
	<i>Aspergillus nidulans</i>	Carter and Bull, 1971
- Shear force	<i>Aspergillus niger</i>	Metz <i>et al.</i> , 1981
	<i>Aspergillus niger</i>	Mitard and Riba, 1988
	<i>Penicillium chrysogenum</i>	van Suijdam and Metz, 1981
	<i>Penicillium chrysogenum</i>	Belmar-Beiny and Thomas, 1991
	<i>Streptomyces clavuligerus</i>	Smith <i>et al.</i> , 1990
- Dilution rate	<i>Fusarium graminearum</i>	Wiebe and Trinci, 1991
- Type of bioreactor	<i>Penicillium chrysogenum</i>	König and Schügerl, 1982
- Shape of cultivation vessel	<i>Penicillium chrysogenum</i>	Hotop <i>et al.</i> , 1989
	<i>Mortierella vinacea</i>	Kobayashi and Suzuki, 1972

2.2 Glucose Oxidase (GOx)

Several microbial enzymes with the capability of oxidizing glucose are known. Out of these, β -D-glucose: oxygen-oxidoreductase (EC 1.1.3.4) is of commercial interest, but in addition there exist several types of glucose dehydrogenases, for example, D-glucose: (acceptor) 1-oxidoreductase (EC 1.1.99.10), D-glucose: NAD(P)⁺ 1-oxidoreductase (EC 1.1.1.118), (EC 1.1.1.119), β -D-glucose: NAD(P)⁺ 1-oxidoreductase (EC 1.1.1.47), and D-glucose (pyrroloquinoline-quinone) 1-oxidoreductase (EC 1.1.99.17), which are also commercially used. Generally, each of these enzymes produces D-glucono-1,5-lactone and a reduced acceptor (Crueger and Crueger, 1990). Glucose oxidase (β -D-glucose: oxygen-oxidoreductase, EC 1.1.3.4) is a FAD dependent glycoprotein catalyzing the oxidation of β -D-glucose to glucono-1,5-lactone. It removes hydrogen from glucose and reduces itself.

The reduced form of GOx is then re-oxidized by molecular oxygen. The developed hydrogen peroxide is decomposed by catalase to water and oxygen giving the net reaction as shown in **Fig. 2.6** (Gibson *et al.*, 1964; Duke *et al.*, 1969).

The kinetics, mechanism of action, properties and molecular structure of GOx were studied by many authors (Swoboda and Massey, 1965; Tsuge *et al.*, 1975; Takegawa *et al.*, 1991; Hecht *et al.*, 1993). Glucose oxidase from *A. niger* is a homodimer with a molecular weight of 150 to 180 kDa. It contains two tightly bound FAD molecules (Pazur and Kleppe, 1964). Dissociation of the sub-units only occur under denaturation conditions and is accompanied by the loss of the cofactor FAD (Jones *et al.*, 1982). The amino acid sequence for the 583 residues protein has been derived from the DNA sequence independently by Kriechbaum *et al.* (1989) and Frederick *et al.* (1990). The enzyme is highly specific for β -D-glucose, with other monosaccharides being oxidized at much lower rate (Adams *et al.*, 1969). Glucose oxidase from *A. niger* is a highly glycosylated protein, the carbohydrate content is ranged from 10 to 24% of its molecular weight (Pazur *et al.*, 1965; Hayashi and Nakamura, 1981). The glycosylated protein contains 190 mannose and 16 *N*-acetyl glucosamine residues. Several functions have been proposed for the carbohydrate moiety of glycoproteins, including correct targeting of proteins, transport through membranes, biological function, immune response and stabilization of the three dimensional structure of the protein (Kalisz *et al.*, 1991). In case of GOx, the deglycosylation did not significantly affect the three dimensional structure of the enzyme. Other properties such as thermal stability, pH and temperature optimum of GOx activity and substrate specificity were not affected. Thus, the carbohydrate moiety of GOx, like that of other glycoproteins, does not appear to contribute significantly to the biological properties of enzyme (Kalisz *et al.*, 1991). On the other hand, the carbohydrate-depleted glucose oxidase was more rapidly precipitated by the addition of trichloroacetic acid and ammonium sulfate than the native enzyme. These results show that the *N*-linked sugar chains of glucose oxidase contribute to the high solubility of the enzyme in water (Takegawa *et al.*, 1989).

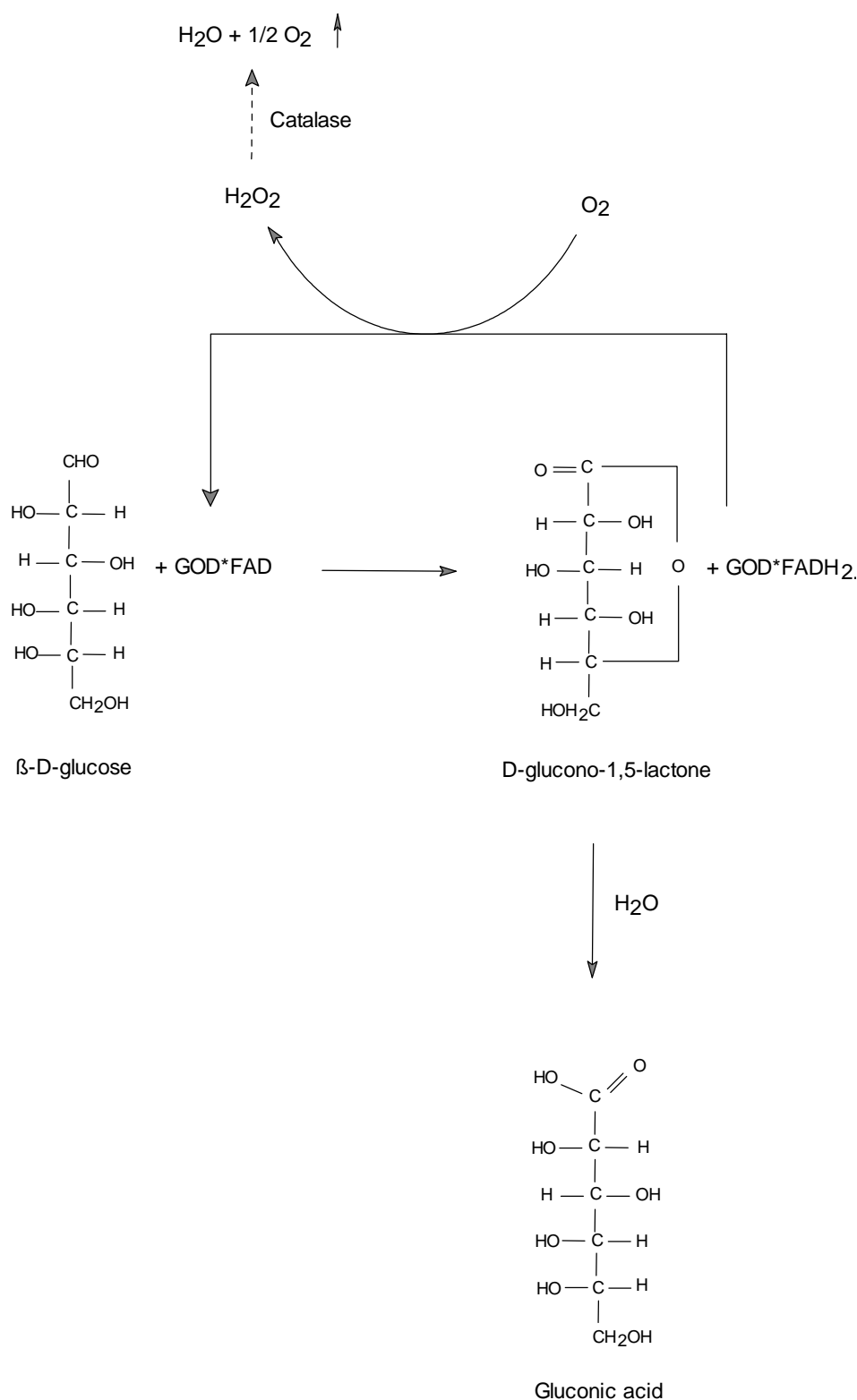


Figure 2.6: Enzymatic conversion of glucose to gluconic acid by glucose oxidase.

2.2.1 Different applications of glucose oxidase

The importance of GOx comes from its wide applications in many fields in crude form, purified form or by using the producer strain.

- In food industries, GOx is used to remove the oxygen from beverages, powdered eggs, and as a source of hydrogen peroxide in food preservation. Moreover, it work as a stabilizer for some food additives such as ascorbic acid and vitamin B₁₂.
- GOx has also a main role in gluconic acid production by *A. niger*.
- In pharmaceutical and analytical biochemistry, it is used for quantitative determination of glucose in biological fluids.
- In manufacturing of glucose biosensors as a new tool for analysis.
- The production of fructose from sucrose can be achieved by treatment with GOx after pre-incubation with invertase. The resulting gluconate can be separated more easily from fructose as it would be the case with glucose.

2.2.2 Glucose oxidase production and excretion

Glucose oxidase was first isolated from a mycelia of *A. niger* and *Penicillium glaucum* by Müller (1928). Nowadays, the industrial production of GOx is carried out using both *A. niger* and *P. amagasakiense*. Beside these two fungi, many other microorganisms were recorded as GOx producers such as *Penicillium variable* (Petruccioli and Federici, 1993), *Phanerochaete chrysosporium* (Kelly and Reedy, 1986), *Talaromyces flavus* (Kim *et al.*, 1990), *Penicillium expansum*, *P. italicum*, and other *Penicillium spp.* (Petruccioli *et al.*, 1993), *Penicillium notatum* and *P. paxilli* (Fiedurek *et al.*, 1986), *Penicillium pinophilum* (Rando, *et al.*, 1997) but in lower concentrations compared to the main industrial producer strains.

Since the first isolation and characterization of glucose oxidase by Müller (1928), much work has been done to optimize the process of GOx production through either genetic manipulation of the host strain or improvement of cultivation conditions. The main studies concerning the production of GOx were carried out using *A. niger*.

Zetelaki and Vas (1968) have investigated the effect of aeration and agitation on the GOx production by *A. niger* in a 5 liter stirred tank bioreactor. They found that the maximum enzyme production was achieved at 700 rpm. Further increases in agitation speed resulted in neither a

higher growth rate nor higher activity. The usage of pure oxygen resulted in an increase of mycelial dry weight of about 1.5 fold and the GOx production was doubled compared to the aerated culture. Zetelaki (1970) found an acceleration of the growth and GOx production of the *A. niger* increased with the sugar consumption in the cultivation broth. Doubling the pressure in the bioreactor (i.e., doubling the solubility of oxygen) resulted in a faster synthesis of enzyme and a higher rate of growth in the early stage of cultivation.

2.2.2.1 Cultivation media for glucose oxidase production

Different media have been used for either *A. niger* cultivation and/or GOx production as reported in **Tab. 2.2**.

Table 2.2: Media for GOD production in submerged culture.

Strain	Medium composition [g l ⁻¹]	Reference
<i>A. niger</i> (RRL 12-6/1-2)	- glucose-monohydrate, 100 ; corn steep liquor (CSL), 20 ; KH ₂ PO ₄ , 1.0 ; MgSO ₄ ·7H ₂ O, 0.25 ; pH 6.5	Traeger <i>et al.</i> , 1991
<i>A. niger</i> N400 (CBS-120-49), mutant	- C-source: glucose, 0.56 M ; fructose, 0.28 M , sodium acetate, 0.1 M ; NaNO ₃ , 1.2 ; KH ₂ PO ₄ , 0.5 ; MgSO ₄ ·7H ₂ O, 0.2 ; YE, 0.5 ; trace metal solution, 0.04 ml. ; pH, 5.5	Witteveen <i>et al.</i> , 1990
<i>A. niger</i>	- glucose, 0.3 M ; KH ₂ PO ₄ , 1.0 ; methylamine hydrochloride, 2.0 ; MgSO ₄ ·7H ₂ O, 0.25 ; K ₂ SO ₄ , 1.0 ; ZnSO ₄ ·7H ₂ O, 0.03 ; FeSO ₄ ·7H ₂ O, 0.03 ; YE, 0.3 ; pH 5.6	Van Dijken and Veenhuis, 1980
<i>A. niger</i> NRRL-3 and mutant	- glucose, 18.0 ; NaNO ₃ , 3.0 ; K ₂ HPO ₄ , 1.0 ; MgSO ₄ ·7H ₂ O, 0.5 ; KCl, 0.5 ; FeSO ₄ ·7H ₂ O, 0.01 ; glycerol, 20.0 .	Markwell <i>et al.</i> , 1989.
<i>A. niger</i> G-13 mutant	- glucose, 80 ; (NH ₄) ₂ HPO ₄ , 0.388 , KH ₂ PO ₄ , 0.188 ; MgSO ₄ ·7H ₂ O, 0.156 , CaCO ₃ , 35 ; pH 5.6	Rogalski <i>et al.</i> , 1988
<i>A. niger</i> NRRL-3 and mutant	- glucose, 80.0 ; NaNO ₃ , 3.0 ; K ₂ HPO ₄ , 1.0 ; MgSO ₄ ·7H ₂ O, 0.5 ; FeSO ₄ ·7H ₂ O, 0.1 ; CaCO ₃ , 35.0 ; pH 7.0	Sharif and Alaeddinoglu, 1992
<i>A. niger</i> 1026/5	- sucrose, 50-70; Ca(NO ₃) ₂ ·4H ₂ O 2.0 ; citric acid, 7.5 ; KH ₂ PO ₄ , 0.25 ; KCl, 0.25 ; MgSO ₄ ·7H ₂ O, 0.25 ; FeCl ₃ ·6H ₂ O, 0.01 ; CSL, 20.0	Zetelaki and Vas, 1968

Table 2.2 cont.

Strain	Medium composition [g l ⁻¹]	Reference
<i>A. niger</i> and other strains	- glucose, 80.0 ; peptone, 3.0 ; (NH ₄) ₂ HPO ₄ , 0.388 ; KH ₂ PO ₄ , 0.188 ; MgSO ₄ · 7H ₂ O, 0.156 ; CaCO ₃ , 35.0	Fiedurek and Szczodrak, 1995
<i>A. niger</i> RRL-12-6/1-2	- glucose-monohydrate, 40.0 ; NH ₄ NO ₃ , 1.0 ; KH ₂ PO ₄ , 1.0 ; MgSO ₄ · 7H ₂ O, 0.25 ; <i>pH</i> 6.5;	Träger <i>et al.</i> , 1992
<i>A. niger</i>	- sucrose, 50.0 ; Ca(NO ₃) ₂ , 2.0 ; citric acid, 7.5 ; KH ₂ PO ₄ , 0.25 ; KCl, 0.25 ; MgSO ₄ · 7H ₂ O, 0.25 ; FeCl ₃ · 6H ₂ O, 0.01 ; CSL, 20.0	Zetelaki, 1970
<i>A. niger</i>	-starch hydrolysate, dextrose basis, 200.0 ; (NH ₄) ₂ HPO ₄ , 0.2 ; CSL, 0.4 ; KH ₂ PO ₄ , 0.1 ; MgSO ₄ · 7H ₂ O, 0.1 ; urea, 0.4 ; antifoam H-601, 0.5 ; <i>pH</i> 6.5	Shah and Kothari, 1993
<i>A. niger</i>	- sucrose, 20.0 ; YE, 10.0 ; peptone, 20.0 ;	Frederick <i>et al.</i> , 1990
<i>A. niger</i> ATCC2029	- glucose, 60.0 ; NH ₄ NO ₃ , 0.3 ; KH ₂ PO ₄ , 0.25 ; MgSO ₄ · 7H ₂ O, 0.25 ; urea, 2.0 ; CSL, 8 ml ; <i>pH</i> , 6.0	Li and Chen, 1994
<i>A. niger</i> and <i>Penicillium</i> spp.	- glucose, 40.0 ; NaNO ₃ , 2.0 ; KCl, 0.5 ; KH ₂ PO ₄ , 1.0 ; MgSO ₄ · 7H ₂ O, 0.5 ; FeSO ₄ · 7H ₂ O, 0.01 , YE, 2.0 ; <i>pH</i> 6.0	Nakamatsu <i>et al.</i> , 1975
	- glucose, 40.0 ; NaNO ₃ , 2.0 ; KCl, 0.5 ; KH ₂ PO ₄ , 1.0 ; MgSO ₄ · 7H ₂ O, 0.5 ; FeSO ₄ · 7H ₂ O, 0.01 ; polypeptone, 3.0 ; <i>pH</i> 6.0	
	- glucose, 60.0 ; NaNO ₃ , 7.0 ; KCl, 0.5 ; KH ₂ PO ₄ , 1.0 ; MgSO ₄ · 7H ₂ O, 0.5 ; polypeptone, 3.0 ; <i>pH</i> 6.0	
	- glucose, 40.0 ; NaNO ₃ , 2.0 ; KCl, 0.5 ; KH ₂ PO ₄ , 1.0 ; MgSO ₄ · 7H ₂ O, 0.5 ; FeSO ₄ · 7H ₂ O, 0.01 ; soyabean meal extract, 5.0 ; <i>pH</i> 6.0	
	- glucose, 50.0 ; meat extract, 10.0 ; polypeptone, 10.0 ; NaCl, 0.5 ; <i>pH</i> 7.0	
<i>P. variable</i> P16.	-glucose, 80.0 ; peptone, 3.0 ; NaNO ₃ , 5.0 ; KCl, 0.5 ; KH ₂ PO ₄ , 1.0 ; FeSO ₄ · 7H ₂ O, 0.01 ; CaCO ₃ , 35.0 ; <i>pH</i> , 6.0	Petruccioli <i>et al.</i> , 1994 Petruccioli <i>et al.</i> , 1995

Table 2.2 cont.

Strain	Medium composition [g l ⁻¹]	Reference
<i>P. variable</i> P16 <i>Penicillium</i> spp.	- glucose, 80.0 ; peptone, 3.0 ; NaNO ₃ , 5.0 ; KH ₂ PO ₄ , 1.0 ; FeSO ₄ ·7H ₂ O, MgSO ₄ ·7H ₂ O, 0.5 ; CaCO ₃ , 35.0	Petruccioli and Federici, 1993
recombinant yeast <i>S. cerevisiae</i>	- sucrose, 80.0 ; YE, 10.0 ; peptone, 20.0	De Baetselier <i>et al.</i> , 1991 De Baetselier <i>et al.</i> , 1992

The C-sources used for GOx production ranged from simple C3 such as glycerol up to a complex C-source such as starch. Glucose was mainly used in all cultivation media with a concentration ranging from 40 to 80 g l⁻¹. However, in industrial scale sucrose or corn steep liquor can be used in case of bulk production of GOx in non purified form which is utilized in gluconic acid production, food preservation and other non-analytical purposes. Also, different organic and inorganic N-sources were added to the cultivation medium to enhance production. Peptone or polypeptone with a concentration ranging from 3 to 20 g l⁻¹, or yeast extract were the main common organic N-sources. On the other hand, sodium nitrate was mainly used as inorganic N-source for GOx production followed by ammonium dihydrogen phosphate. The source of phosphate was in the form of either potassium dihydrogen phosphate or dipotassium hydrogen phosphate with a concentration ranging from 0.2 to 1.0 g l⁻¹. Other trace inorganic salts for supplementation with Mg⁺⁺, Fe⁺⁺, Zn⁺⁺, and K⁺ cations were also added in smaller amounts. Moreover, either magnesium carbonate or calcium carbonate were used in some publications in case of cultivation in shake flask to neutralize the acidity of the cultivation medium due to gluconic acid production which is concomitant with GOx production on using glucose as C-source.

Nakamatsu *et al.* (1975) studied the effect of different complex carbon sources as well as different nitrogen sources on GOx production. Among eight sources of widely differing natural carbon sources, they found that beet molass was the best carbon source to support growth and GOx production. On the other hand, nitrate and urea gave a better GOx yield than ammonium salts.

Under normal condition of glucose oxidase production with *A. niger*, glucose oxidase behaves as an intracellular enzyme and it is released from the mycelium by means of cell disruption techniques. van Dijken and Veenhins (1980) used cytochemical staining technique to establish the location of GOx in microbodies. Mischak *et al.* (1985) have shown that at low pH values *A. niger* produce citric acid while after the adjustment of pH to 5.5 a *de novo* synthesis of GOx occurs and most of the GOx produced is secreted. The level of GOx produced by the above pH shift method was lower than that are obtained in fermentation process purposed for GOx production.

Ishimori *et al.* (1982) have described an interesting approach to dissolve the problem of enzyme release from fungal cells. Mycelia of a GOx producer strain of *A. niger* were cultivated in a small bioreactor fitted with an ultrasonic generator and grown under mild sonication condition. Some of the GOx was released into the medium without disruption of the cells, however, the mechanism of this release is not clear.

The production of GOx was also carried out successfully using immobilized cells. Fiedurek and Ilczuk (1991) studied the GOx production by *A. niger* immobilized on sintered glass, rasching rings, pumice stones and polyurethane foam for extracellular GOx production. The GOx produced by the immobilized cells was 2.7 times higher than that of free cells. Petruccioli *et al.* (1994) studied the GOx production by *Penicillium variable* P16 immobilized on/in different carriers. Among different carriers, polyurethane proved to be the best for GOx production and the production continued for 7 repeated batches. A new method for GOx production by *A. niger* conidia immobilized on seeds was also studied by Fiedurek *et al.* (1994). The adsorption of *A. niger* spores on wheat seeds is a very simple and inexpensive method of immobilization and continued production of GOx was observed for 8 repeated batches.

2.2.2.2 Glucose oxidase production by recombinant microorganisms

However, the natural host organism did not prove to be ideal for GOx over-expression. First, GOx expression and excretion depends on the presence of glucose and pH of the culture medium higher than 4.5. GOx production leads to gluconic acid and hydrogen peroxide production which complicates the cell culture techniques. These problems can probably be circumvented by substituting the natural GOx promoter with another potent and glucose-independent *Aspergillus* promoter (Kopetzki *et al.*, 1994). Second, GOx is usually cell associated, thus making purification more difficult. Third, purified *Aspergillus* GOx is often contaminated with host cell enzymatic impurities such as catalase, amylase and cellulase which interfere with its applications. To overcome these disadvantages, GOx expression and secretion were studied in *Saccharomyces cerevisiae* (De Baetselier *et al.*, 1991). The mature GOx coding sequence was fused to the yeast α -factor prepro-sequence or the natural GOx signal sequence and then inserted into episomal replicating 2- μ m DNA-based multicopy vectors under the control of a tightly regulated yeast promoter (Frederick *et al.*, 1990). However, the yeast derived GOx was very heterogeneous in size compared to GOx obtained from *A. niger*. The application of yeast derived GOx is restricted to food processing and not accepted as a substitute for the *A. niger* derived enzyme in established diagnostic application, mainly because of the reduced specific activity (Kopetzki *et al.*, 1994).

The production of GOx by recombinant strains has been carried out to improve secretion of GOx into the medium (Hellmuth *et al.*, 1995). Principally, two main strategies for the over-expression of homologous genes are possible:

- 1- Amplification of the gene copy number.
- 2- Expression of the gene under the control of strong regulatory elements.

In the first case, a filamentous fungus is transformed with an isolated gene encoding the protein of interest. Several gene copies will integrate stably into the genome. This genome amplification causes an enhancement in protein production. The best characterized strongly expressed fungal gene encode the glucoamylase structure gene of *A. niger*, the constitutive promoter of strongly expressed glyceraldehyde-3-phosphate dehydrogenase gene (*gpdA*) of *Aspergillus nidulans* and the α -amylase signal sequence of *A. oryzae* was studied by Urmann

et al. (1992). However, this method of high level expression of enzyme in *A. niger* NRRL-3 was also studied for pectin methyl esterase production (Khanh *et al.*, 1992). The combination of *gpdA* promoter with the α -amylase signal sequence could also improve the homologous protein secretion and GOx production in a recombinant *Aspergillus niger* NRRL 3 (GOD 3-18), Hellmuth *et al.* (1995).

3. MATERIALS AND METHODS

3.1. Materials:

3.1.1 Chemicals and Enzymes

-ABTS [®]	Boehringer Mannheim
2,2'-Azino-di-[3-ethylbenzthiazolin sulfonate],	
-Acetone	Merck
-Acridine orange	Merck
-Agar	Difco
-Ammonium-heptamolybdate	Merck
-Ammonium-phosphate	Merck
-Ammonium-sulfate	Merck
-Arabinose	Sigma
-Ascorbic acid	Merck
-Bacto peptone	Difco
-Citric acid	Sigma
-O-Dianisidin	Riedel de Haën
-DNS, (3,5-dinitrosalicylic acid)	Sigma
-Ferrous sulfate	Riedel de Haën
-Fructose	Merck
-Galactose	Sigma
-Gluconic acid	Sigma
-Glucose	Merck
-Glycerol	Merck
-Horse radish peroxidase	Boehringer Mannheim
-Magnesium sulfate	Riedel de Haën
-Malt extract	Fluka
-Mannose	Sigma
-Oxalic acid	Sigma
-Potassium chloride	Merck
-Potassium hydrogen phosphate (dibasic)	Merck

-Rhamnose	Sigma
-Ribose	Sigma
-Sodium nitrate	Riedel de Haën
-Sulfuric acid	Merck
-Urea	USB
-Xylan	Roth
-Xylitol	Sigma
-Xylose	Fluka
-Yeast extract	Difco

3.1.2 Microorganism

The cell physiological studies were carried out using genetically modified strain, *A. niger* NRRL-3 (GOD 3-18), Hellmuth *et al.* (1995). This strain carries a glucose oxidase structural gene of the wild type strain fused to the α -amylase signal sequence of *A. oryzae*. The expression of this recombinant glucose oxidase gene is controlled by the glyceraldehyde-3-phosphate dehydrogenase promoter (*gpdA*) of *A. nidulans* [Fig. 3.1].

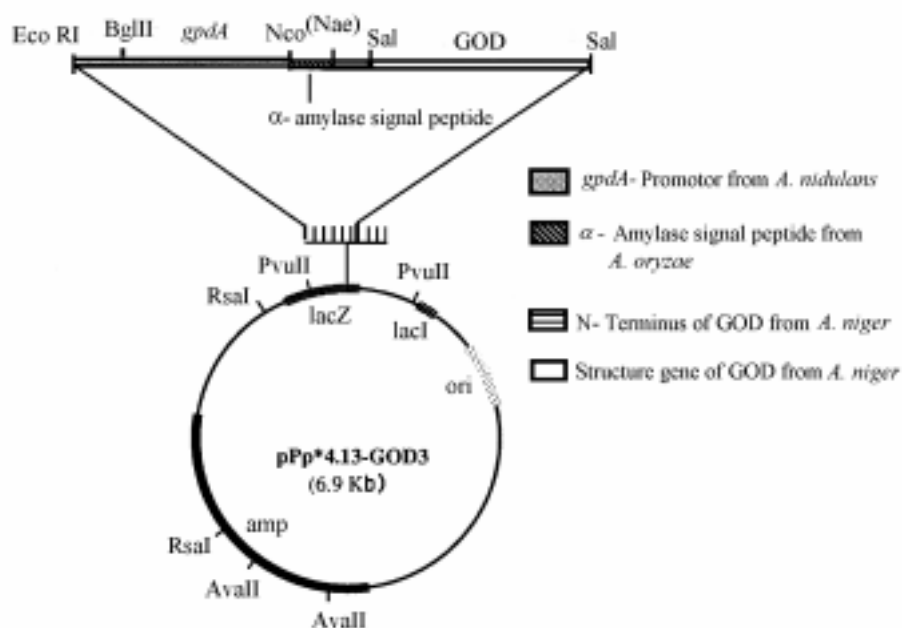


Figure 3.1: Transformation vector for the construction of *Aspergillus niger* NRRL-3 (GOD 3-18). (J.K. Jung and E. Ruttkowski, Technische Hochschule Darmstadt).

3.2 Methods

3.2.1 Inoculum preparation

As stock culture, spore suspension stored at -80°C in 50% glycerin was used. Using this method of strain preservation, neither change in cell productivity nor morphology were observed throughout this study. Unless otherwise stated, inoculation was carried out for both shake flask and bioreactor by 1×10^7 spores ml^{-1} obtained from a densely conidiating culture grown on CM agar medium for 48-72 h. The spores were harvested with a sterile physiological saline solution, NaCl (0.9 % w/v), and counted using a haemocytometer slide.

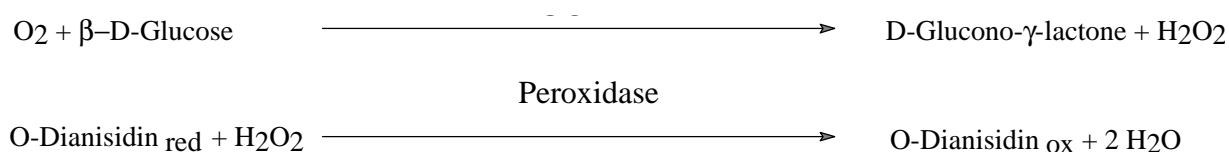
3.2.2 Cultivation media

The agar plate medium, CM-medium, for sporulation of the recombinant strain *A. niger* is composed of

Malt extract	20.0 g
Bacto peptone	1.0 g
Glucose	20.0 g
Agar	20.0 g
Distilled H_2O	1000 ml

The final pH was adjusted to 5.5 before sterilization.

Glucose oxidase positive strain was identified on agar plate containing 0.1 g l^{-1} O-Dianisidin and 6000 U ml^{-1} of horse radish peroxidase (Boehringer Mannheim). If GOx is formed, the following enzymatic reaction will occur giving rise a brown color.



Unless otherwise stated, the medium used for glucose oxidase production was composed of

NaNO ₃	3.0 g
K ₂ HPO ₄	1.0 g
MgSO ₄ ·7H ₂ O	0.5 g
KCl	0.5 g
FeSO ₄ ·7H ₂ O	0.01 g
Yeast extract	2.0 g
C-source (glucose, etc...)	80.0 g
Distilled H ₂ O	1000 ml

The final pH was adjusted to pH 5.5 before sterilization, glucose was sterilized separately and added to the medium before inoculation.

In case of fed-batch cultivation, the composition of starting and feeding medium was as follows:

component	Starting medium [g l ⁻¹]	Feeding solution [g l ⁻¹]
Xylose	40.0	200.0
NaNO ₃	3.0	57.0
K ₂ HPO ₄	1.0	19.0
MgSO ₄ ·7H ₂ O	0.5	-
KCl	0.5	8.5
FeSO ₄ ·7H ₂ O	0.01	0.19
Yeast extract	2.0	(added step-wise)

The feeding was carried out according to the method of Korz *et al.* (1992) as follows:

$$m_s(t) = \left[\frac{1}{Y_{X/S}} \cdot \mu_{set} + m_E \right] \cdot V_L(t) \cdot X_F \cdot e^{\mu_{set}(t-t_F)} \quad [3.1]$$

where

m_s	Mass flow of substrate [g h^{-1}]
t	Cultivation time [h]
t_F	Start time of feeding phase [h]
μ_{set}	Adjusted specific growth rate [h^{-1}]
m_E	Maintenance coefficient [$\text{g g}^{-1} \text{h}^{-1}$]
$Y_{X/S}$	The biomass/substrate yield coefficient [g g^{-1}]
X_F	The biomass concentration at the start time of feeding phase [g]
V_L	The culture volume [L]

3.2.3 Cultivation conditions

3.2.3.1 Shake flask cultivation

Shake flask experiments were carried out in two types of 100 ml Erlenmeyer flasks (with 2 baffles and without baffles). Cultivation was carried out on a rotary shaker (Pilot Shaker Rc 6SR, B. Braun Diesel Biotech GmbH, Melsungen, Germany) at 120 rpm and 30°C, and 20 ml working volume.

3.2.3.2 Bioreactor cultivation

Two types of bioreactors, 2-L and 5-L, were used in this study.

Cultivation in 2-L bioreactor

A stirred tank bioreactor SGi- Set 2 (SGi, Rouillac, France) with a working volume of 1-L was used. Agitation was performed using a single 4-bladed rushton turbine impeller at 200-600 rpm. (details are given in the experimental part of this work). The initial pH was adjusted to 5.5 and controlled during the cultivation using 2.5 mol L^{-1} NaOH.

Cultivation in 5-L bioreactor

Cultivations were also carried out in 5-L stirred-tank bioreactor Biostat MD (B. Braun Diesel Biotech. GmbH, Melsungen, Germany) with a working volume of 3 liters. **Fig. 3.2** shows the schematic structure of this bioreactor and its geometric dimensions.

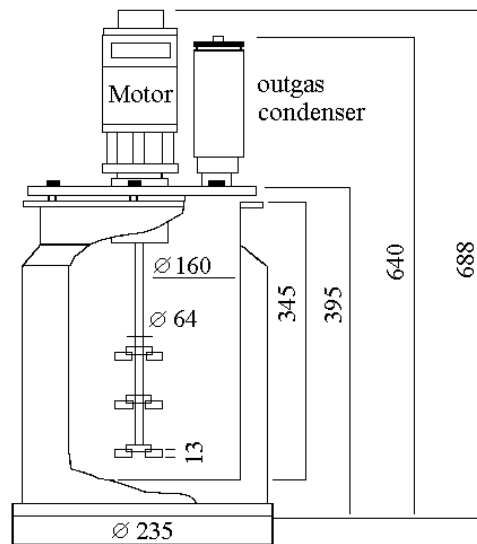


Figure 3.2: 5-L Stirred tank bioreactor (MD, B. Braun, Melsungen)

The stirrer of the Biostat MD was equipped with three 6-bladed rushton turbine impellers ($d_i = 64 \text{ mm}$, $d_t = 160 \text{ mm}$, $hd_t^{-1} = 1.0$, $d_i d_t^{-1} = 0.4$). Unless otherwise stated, the agitation was carried out at 200 rpm for the first 5 hours of the cultivation and increased up to 800 rpm for the rest of cultivation. Aeration was performed by filtered sterile air (0.75 L min^{-1}), during the

early 5 hours of cultivation and increased to 1.5 L min^{-1} afterwards). The decreased agitation and aeration in the first hours was performed to prevent spore flotation and adhesion to the walls of the culture vessels. The pH was controlled at 5.5 by addition of 2.5 mol L^{-1} NaOH. Foam was suppressed, when necessary, by the addition of antifoam reagent SP1 (Th. Goldschmidt AG, Essen, Germany). The digital control unit of the Biostat MD bioreactor, mass flow meter, balance of NaOH solution, pumps and exhaust-gas analysis system were interfaced to a VME-bus microcomputer using UBICON (Universal Bio-Process Control System) software (Bellgardt *et al.*, 1992) for data acquisition and processing.

The concentration of oxygen and carbon dioxide in the exhaust gas of bioreactor cultures were determined by paramagnetic and infrared gas analysis systems, respectively (Maihak, Germany). Dissolved oxygen concentrations were analyzed by polarographic electrode (Ingold, Germany). Aeration rate and off-gas analysis data were used to calculate the oxygen uptake $Q \text{ O}_2$ and carbon dioxide production rates $Q \text{ CO}_2$ ($\text{mmol L}^{-1} \text{ h}^{-1}$) and the respiratory quotient assuming steady-state conditions between gaseous and liquid phases. **Fig. 3.3** shows the Biostat MD bioreactor with all connected instruments as used in this study.

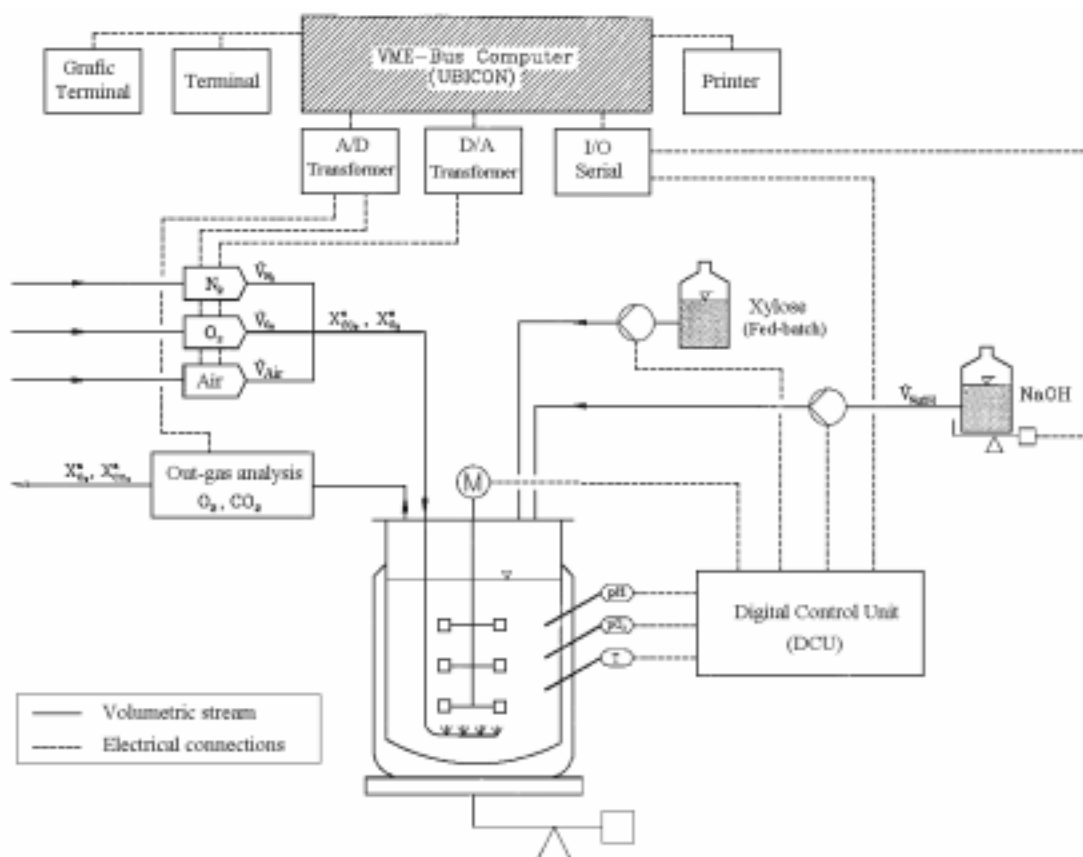


Figure 3.3: A schematic diagram of the 5-L bioreactor (Biostat MD)

3.2.4 Calculation of oxygen uptake rate and carbon-dioxide formation rate

Oxygen uptake rate and carbon dioxide production rates and the respiratory quotient (RQ) were calculated according to the as **Eqs. 3.2-3.4** as follows:

$$Q_{O_2} = \frac{V_G * p}{R * T * V_L} * \left[x^e_{O_2} - x^a_{O_2} * \frac{(1 - x^e_{O_2} - x^e_{CO_2})}{(1 - x^a_{O_2} - x^a_{CO_2})} \right] \quad [3.2]$$

$$Q_{CO_2} = \frac{V_G * p}{R * T * V_L} * \left[x^a_{CO_2} * \frac{(1 - x^e_{O_2} - x^e_{CO_2})}{(1 - x^a_{O_2} - x^a_{CO_2})} - x^e_{CO_2} \right] \quad [3.3]$$

$$RQ = \frac{Q_{CO_2}}{Q_{O_2}} \quad [3.4]$$

with:

Q_{O_2}	Volumetric oxygen uptake rate mmol L ⁻¹ h ⁻¹
Q_{CO_2}	Volumetric carbon dioxide production rate mmol L ⁻¹ h ⁻¹
V_G	Air flow L h ⁻¹
p	working pressure (1 atm)
R	Gas constant 8.2057*10 ⁻⁵ l atm mmol ⁻¹ K ⁻¹
T	Absolute temperature, K
V_L	Volume of liquid phase, L
$x^e_{O_2}$	Mole fraction of oxygen in inlet air
$x^e_{CO_2}$	Mole fraction of carbon dioxide in inlet air
$x^a_{O_2}$	Mole fraction of oxygen in off gas
$x^a_{CO_2}$	Mole fraction of carbon dioxide in off gas

3.2.5 Sample preparation and biomass determination

In case of shake flask cultures, samples in the form of 3 flasks each were taken after regular time interval during cultivation. During bioreactor cultivation, aliquots of the culture were removed from the vessel through a sampling tube of 4 mm diameter, whose open end was approximately 4 cm above the base of the cultivation vessel. The sample (usually 10 ml) was withdrawn using a syringe acting through an in-line air filter.

Samples were collected in preweighed centrifugation tube of 15 ml. (Falcon, USA), centrifuged at 5 °C with 5000 rpm (1550× g) for 20 min (Heraeus Omnifuge 2.0RS, Heraeus, Germany) and supernatant was filtered (0.45 µm, Ministar NML, Sartorius). A small fraction of sample was frozen at -80 °C and used for sugar, GOx and organic acid determination. The cell pellets were washed twice using bi-distilled water, centrifuged again and were either frozen immediately at -80 °C until they were used for intracellular enzyme determination or dried in an oven at 40 °C under vacuum for determination of cell dry weight (Salvis KVTS 11, Salvis AG, Switzerland).

3.2.6 Cell disintegration for intracellular enzyme determination

For intracellular enzyme determination, a mechanical cell disruption using a vibration mill (Perkin Elmer 053257) was employed. Biomass samples, stored at -70 °C, were thawed in 10 ml sodium phosphate buffer (30 mM, pH 5.0) and collected on filter paper (Schleicher and Schuell, Germany). The wet fungal cells were transferred into the mill chamber (chrome steel, length 30 mm, diameter 12 mm) containing two steel spheres (diameter 5 mm). Cell disruption was completed in a vibration time of 2.5 minutes at a vibrating frequency of 50 Hz. In intervals of 30 seconds, the mill chamber was cooled over liquid nitrogen. The chamber content was resuspended in 3 ml sodium phosphate buffer (30 mM, pH 5.5) and immediately used for intracellular GOx activity measurement.

3.2.7 Analysis of cultivation broth

3.2.7.1 Determination of carbohydrates and organic acids

HPLC method

The quantitative determinations of carbohydrates (glucose, fructose and xylose), organic acids (gluconic acid, citric acid and oxalic acid) as well as xylitol were carried out by HPLC using an Aminex HXP-87H column (BioRad) for separation ($T = 25^{\circ}\text{C}$) and ultraviolet (UV) and refractive index detector (Techlab, Germany) for detection. Sulfuric acid (5 mmol) was used as mobile phase (flow rate = 0.5 ml min^{-1}). Beside HPLC method, other methods were also used for determination.

Chemical and biochemical methods

Determination of glucose

Glucose concentration was determined using glucose analyzer (Yellow Springs, Model 2000), (Yellow Springs, Ohio, USA).

Determination of xylose

Xylose concentrations were determined by a colorimetric method as described by Miller (1959), summarized in **Fig. 3.4**. The chemical method is based on the determination of the colour developed after the reaction between the reduced sugar and DNS (3,5- Dinitrosalisyllic acid) in citrate buffer solution.

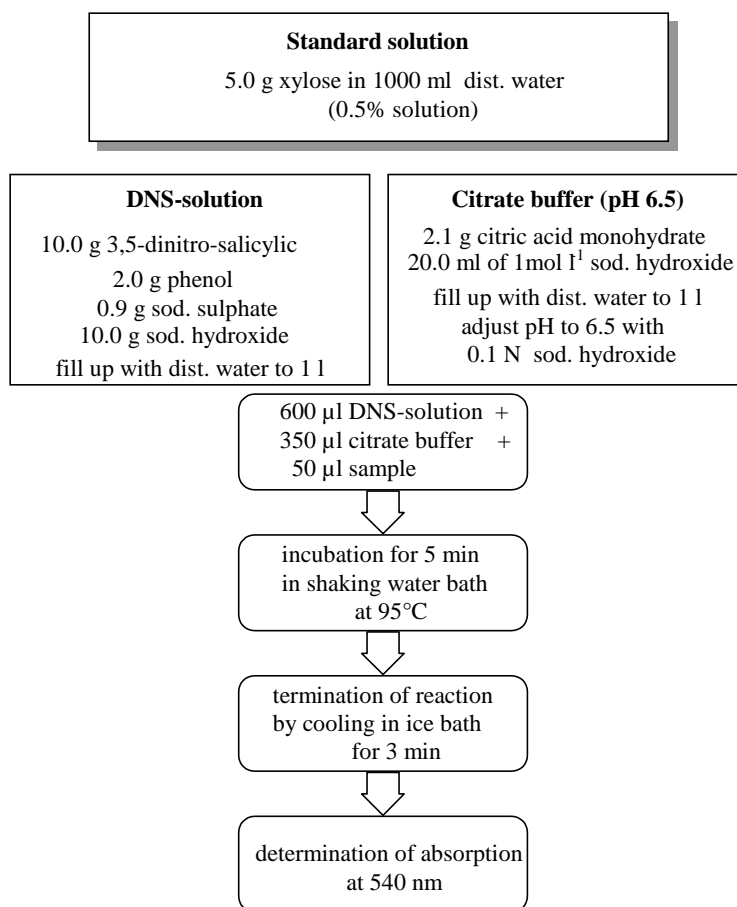


Figure 3.4: Colorimetric method for xylose determination.

Determination of gluconic acid

Beside quantitative determination using HPLC as described before, gluconic acid concentration was also determined enzymatically using gluconic acid determination kit (Boehringer Mannheim, Cat. No. 428 191).

3.2.7.2 Determination of phosphate

Ortho-phosphate ions in the cultivation broth were determined spectrophotometrically at 660 nm according to the method described by Boltz (1972) as summarized in **Fig. 3.5**. This method is based on the reaction between ammonium-heptamolybdate and o-phosphate in an acidic medium causing the appearance of a blue colored complex.

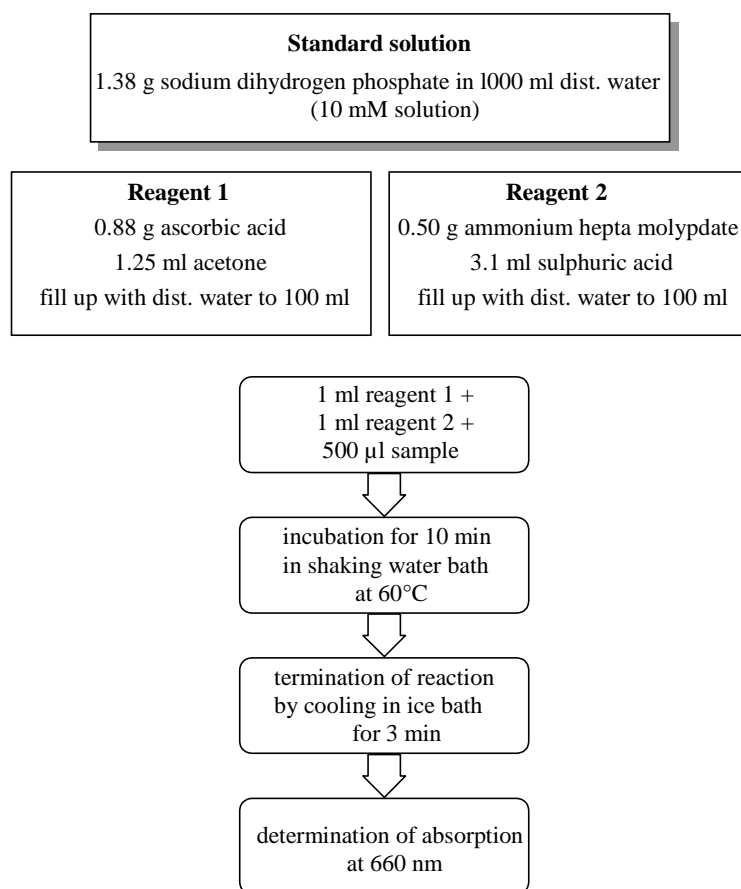


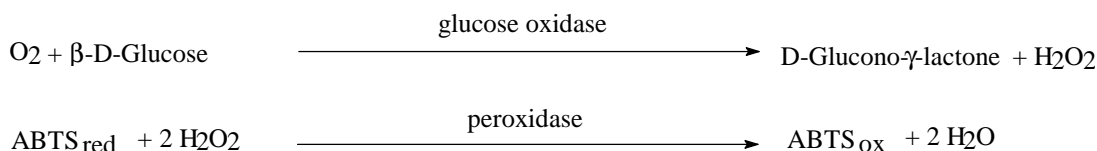
Figure 3.5: Colorimetric method of phosphate determination in culture medium.

3.2.7.3 Determination of nitrate

The quantitative determination of nitrate ion in the cultivation broth was carried out using a nitrate ion selective electrode (9300BN, Orion 93-07, Orion, Boston, USA) and a double junction reference electrode (Orion 90-02, Orion, Boston, USA). The electrodes were connected to a microprocessor pH-meter (761 Calimatic, Knick, Berlin, Germany). A standard curve was obtained using different concentrations of nitrate solution. For both standard and test sample, diluted nitrate-ISA-solution (nitrate ionic strength adjuster) composed of ammonium-sulfate was used to stabilize the conductivity of solution.

3.2.7.4 Determination of glucose oxidase activity

The quantitative determination of glucose oxidase (GOx) was carried out indirectly by spectrophotometric method according to the following reaction:



ABTS[®] : 2,2'-Azino-di-[3-ethylbenzthiazolin-sulfonat], (Boehringer Mannheim, Cat. No. 75640)

In the first reaction, glucose oxidase forms hydrogen peroxide which is subsequently used to oxidize ABTS[®] in the second reaction by peroxidase (Horse radish peroxidase, Boehringer Mannheim Cat. No. 108 073). ABTS[®] forms a reversible redox system in which the oxidized form has a characteristic bluish green color of a stable radical cation. The experimental method for GOD determination was carried out as follows: 800 μl of a freshly prepared, oxygen saturated solution of 315 mmol glucose, 1.25 mmol ABTS[®] and 111 mmol sodium phosphate buffer solution of pH 5.8 was added to 100 μl peroxidase solution (horse radish peroxidase, Boehringer 108 073 , 10 Unit ml^{-1} in 111 mM sodium phosphate buffer pH 5.8) and 100 μl of appropriated diluted sample. The optical absorption of the mixture was determined spectrophotometrically at a wave length of 420 nm and 25°C for four minutes (LKB Biochrom, Ultrospeck 4054).

The glucose oxidase activity was calculated according to **Eq. 3.5**.

$$A = \frac{d [\text{Glucose}]}{dt} = \frac{d [\text{ABTS}]}{dt} = \frac{f}{\epsilon * l} * \frac{dE}{dt} \quad [3.5]$$

with

A: Activity of glucose oxidase in the sample

$$[A] = \mu\text{mol sec}^{-1} \text{ ml}^{-1} = \text{nkatal ml}^{-1}$$

$\frac{dE}{dt}$	The time dependent increase in absorbance at 420 nm. $dE/dt = \text{sec}^{-1}$
f :	dilution factor of the sample
l :	the path-length of the cuvette, [l] = cm.
ε :	Extinction coefficient of ABTS [®] at 420 nm. $\varepsilon = 43.20 \text{ ml } \mu\text{mol}^{-1} \text{ cm}^{-1}$ (Bergmeyer, 1983).

The minimal amount of enzyme detected by this method was $0.17 \text{ nkat ml}^{-1}$. The accuracy of this method is of about $\pm 5 \%$.

3.2.8 Morphological studies

3.2.8.1 Staining of bioparticles

Acridine orange (AO) was used for staining of all types of bioparticles. AO forms a green fluorescence complex with double strand RNA and DNA and red fluorescent complex with single strand RNA. A sample taken from the bioreactor was directly fixed by ethyl alcohol [96%] to stop the metabolic activities and followed by fixation on microscopic slide. Fixation was carried out for 60 min at 80°C . The fixed samples were stained using AO-stock solution (400 μmol acridin orange in 0.1 mol Na-phosphate buffer, pH 7.0). After 2 min, the dye solution was removed by washing with distilled water and the samples were allowed to dry again at room temperature. The green and red fluorescence were observed using a fluorescence microscope (Leica DM LB, Germany) with a I-3 filter.

3.2.8.2 Quantification and qualification of different types of bioparticles*

(*, Any discrete biomass in a culture was considered as `bioparticle`; particles may consist of a single spore, an aggregation of spores or fungal pellet.).

Method 1: large pellets (> 3 mm diameter), in shake flask culture

A sample of the suspension was transferred to a petri dish and a macrophoto was taken. The diameter of pellets on the photograph was measured using a vernier caliper. Calibration was achieved by means of a standard millimeter scale, photographed together with the pellets. Pellet diameters were measured (of at least 100 samples) and the mean value was estimated.

Method 2: small bioparticles (< 3 mm diameter)

For small bioparticles (spores, aggregates and small pellets) obtained during bioreactor cultivation, measurements were carried out using a PC-based system and adequate Image Analysis (Leica Q500MC, Leica, Germany). Images were captured via a colored video camera (CCD-colored video camera CF 15/4 MCC, Kappa, Germany) connected to a microscope (Leica DM LB, Germany) via an image control unit MCU/C [Fig. 3.6]. The maximal magnification of this unit is $\times 1000$. For both spores and small aggregates, the average diameter for each sample was measured manually (by touching two opposite points using a mouse).

The microscopic magnification was set at $\times 1000$, and the bioparticles were selected randomly. In all cases, the data represented in this study are an average of randomly chosen 100-120 bioparticles. Photographs were taken with a Zeiss Photomicroscope (Axioskop).

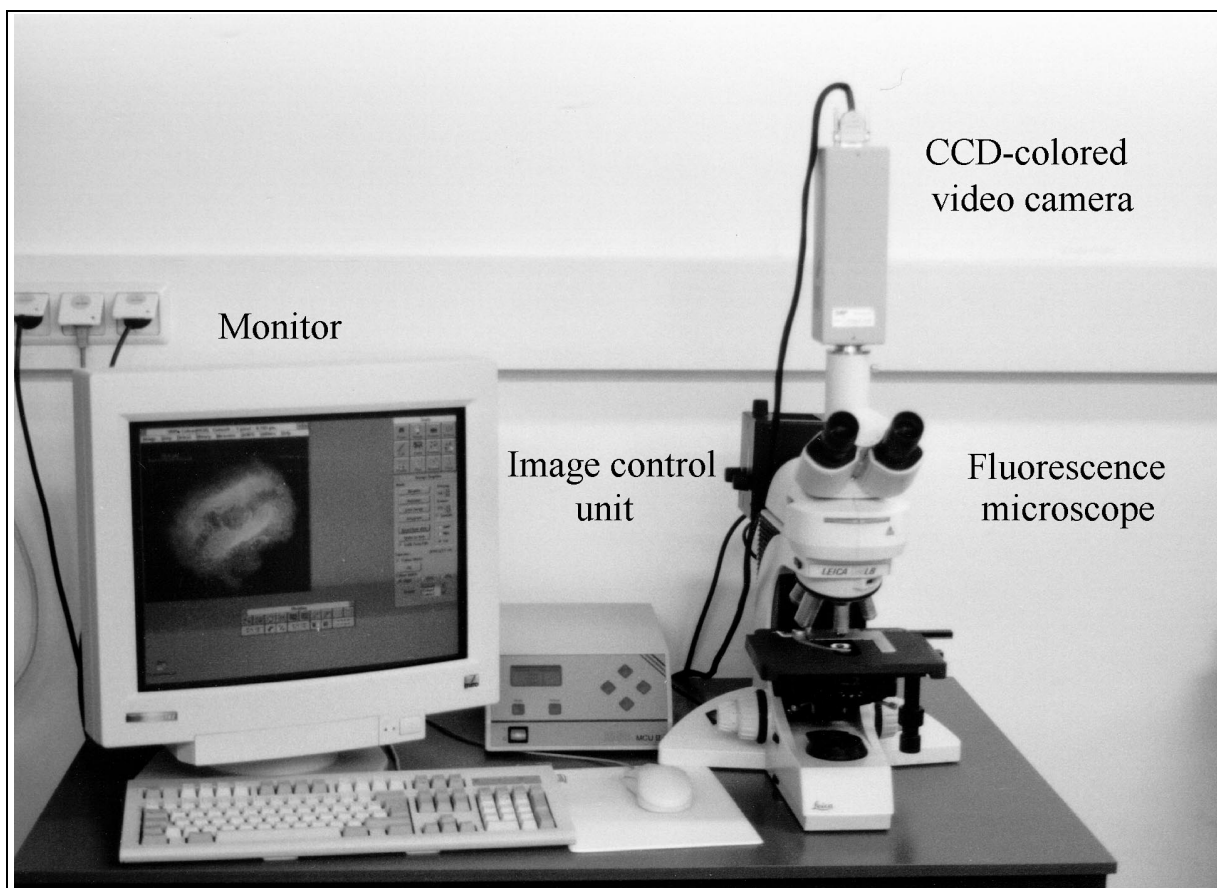


Figure 3.6: Fluorescence microscope with image analysis system.

3.2.8.3 Measurement and Calculations

The average diameter for each bioparticle and length in case of germ tube was measured manually by touching two opposite points on the object under study. The microscope magnification was set at $\times 50$ - $\times 200$ (in case of pellet measurement) or at $\times 400$ - $\times 1000$ (in case of spore, germ tube and hyphae measurement). For each sample, the process was repeated at least 100 times using new positions on the same and on different bioparticles.

The microscopic morphology, that is the average total hyphal length ($l_{h,av}$), and the average diameter (D_h) of the hyphal element, has been quantified during batch cultivation. Assuming that the water content and the density in hyphal element is constant, that the hyphal diameter is constant and that there is no fragmentation, the specific hyphal extension rate (μ_h) was measured according to Carlsen *et al.* (1996) as follows:

$$\frac{dl_{h,av}}{dt} = \mu_h \cdot l_{h,av} \quad [3.6]$$

where $dl_{h,av}/dt$, the average hyphal extension rate and $l_{h,av}$ is the average of hyphal length

Determination of bioparticle volume

A. In case of spore

The spore volume can be calculated using **Eq. 3.7** which assume the spores are spherical and their two-dimensional projections circular:

$$V_s = \frac{\pi \cdot D_s^3}{6} \quad [3.7]$$

where D_s is spore diameter and V_s is spore volume.

B. In case of germ tube and hyphae

In case of germ tube and hyphae, hypha is regarded as a solid cylinder and the volume of the germ tube or hyphae can be calculated according to the following equation:

$$V_h = (\pi \cdot D_h^2 \cdot l_h) \times 0.25 \quad [3.8]$$

where V_h is hyphal volume; D_h hyphal diameter and l_h hyphal length

Determination of spore swelling and hyphal extension rates

For determination of spore swelling kinetic, the average spore diameter (D_s) was followed during the early time of cultivation (0-10 h) and the specific spore swelling rate (μ_{sv}) was determined as follows:

$$\frac{dV_s}{dt} = \mu_{sv} \cdot V_s \quad [3.9]$$

The specific increase in total bioparticle volume (μ_{bp}) was determined as follows:

$$\frac{dV_{bp}}{dt} = \mu_{bp} \cdot V_{bp} \quad [3.10]$$

where v_{bp} is the total bioparticle volume (spore volume + hyphal volume(s)).

Calculation of active biomass in case of growth in pellet form

Calculation of the percentage of active fraction of cells in case of growth in pellet form was carried out as follows:

$$CDW_{total} = CDW_{active} + CDW_{inactive} \quad [3.11]$$

$$CDW_{active} = V_{active} \cdot \rho \quad [3.12]$$

$$V_{total} = V_{active} + V_{inactive} \quad [3.13]$$

$$V_{total} = \frac{4}{3} \pi \cdot \left(\frac{D_{total}}{2} \right)^3 \quad [3.14]$$

$$D_{total} = D_{active} + D_{inactive} \quad [3.15]$$

$$D_{active} = D_{total} - D_{inactive} \quad [3.16]$$

$$D_{inactive} = D_{total} - 2w \quad [3.17]$$

w : The thickness of active layer in μm (the growing active zone which gives a red color under fluorescence microscope after staining with AO). The thickness of this active layer was varied according to the cultivation conditions.

In case of bioparticles smaller than 2 w

The active pellet volume is equal to the total volume and there is no mass transfer limitation in this type of pellet. The active volume of pellet can be directly obtained from **Eq. 3.14** where:

$$V_{total} = V_{active}$$

In case of bioparticles larger than 2 w

The active bioparticle volume can be calculated through the following equation

$$V_{active} = V_{total} - V_{inactive} \quad [3.18]$$

$$V_{active} = \left(\frac{4}{3} \pi \cdot \left(\frac{D_{total}}{2} \right)^3 \right) - \left(\frac{4}{3} \pi \cdot \left(\frac{D_{total} - 2w}{2} \right)^3 \right) \quad [3.19]$$

The percentage of active volume fraction was calculated as follows:

$$V_{active} [\%] = \frac{V_{active} ml^{-1}}{V_{total} ml^{-1}} \cdot 100 \quad [3.20]$$

Consequently the percentage of active fraction of cell mass was calculated assuming that there are no differences in cell density inside the pellet in bioreactor cultures).

Note:

(The total volumes of pellets were calculated as a sum of different volume fractions in population, where the intra-population variation was high in pelleted grown culture).

4. Results and Discussion

The aim of this work was to improve GOx production and excretion using a recombinant *A. niger*. The first part of this study was done to improve this process through the change in medium composition and to find out the relation between cell morphology and GOx production in glucose culture by using different cultivation vessels (baffled, non-baffled shake flasks and 2-L and 5-L bioreactors). Then studies were oriented to GOx production with non-glucose C-sources in both shake flask and bioreactor levels. For further studies on the relation between the growth morphology and GOx production and excretion under different hydrodynamic stress, cultivations were done in stirred tank bioreactor under different agitation speeds. Further studies were also done to the improvement of fungal morphology and GOx production through the addition of a biopolymer (xylan) to the cultivation medium. Finally, this process was further improved by fed-batch cultivation to increase the cell mass and GOx production.

4.1. Cultivation of a recombinant *A. niger* in glucose cultures

4.1.1. Shake flask cultivations

During the preliminary experiments, different studies were done to change the constituents and concentrations of the cultivation medium through utilization of different sources and concentrations of N-source, P-source, etc. The optimal production was achieved using 3 g l⁻¹ sodium nitrate. On the other hand, phosphate did not show any significant effect on GOx production and more than 50% of phosphate concentration remained non-utilized until the end of cultivation time. However, the most important constituent of this medium was yeast extract.

4.1.1.1. Effect of yeast extract addition on fungal morphology and extracellular glucose oxidase production

The effect of the addition of different yeast extract concentrations to the basic minimal medium on fungal growth, extracellular GOx production and fungal morphology was studied in shake flask cultures [Fig. 4.1].

The results show that the addition of increasing concentrations of yeast extract to the basic minimal medium increased the final biomass concentration concomitantly. However, an increase in the final

extracellular GOx activity (GOx_{ext}) occurred only when yeast extract was added to the medium in concentrations up to $1\text{--}2\text{ g l}^{-1}$. Increasing the concentration of yeast extract to more than 2 g l^{-1} resulted in decreasing extracellular GOx activities declining to non-detectable levels with the addition of yeast extract in concentrations above 6 g l^{-1} . An investigation of the fungal morphology revealed a change from a filamentous to a pelleted form upon the addition of yeast extract to the basic minimal medium. Further studies on the fungal morphology showed that increasing concentrations of yeast extract caused the fungal pellets to increase in size but decrease in density and number per unit volume [Fig. 4.1B].

The stimulatory effect of yeast extract in concentrations up to $1\text{--}2\text{ g l}^{-1}$ on the extracellular production of recombinant GOx in shake flask cultures can be attributed to the presence of amino acids, vitamins and/or other compounds in this complex organic substrate. However, this effect of the complex organic substrate on the extracellular production of GOx is apparently abolished with the addition of higher concentrations of yeast extract that caused the formation of bigger and less dense pellets.

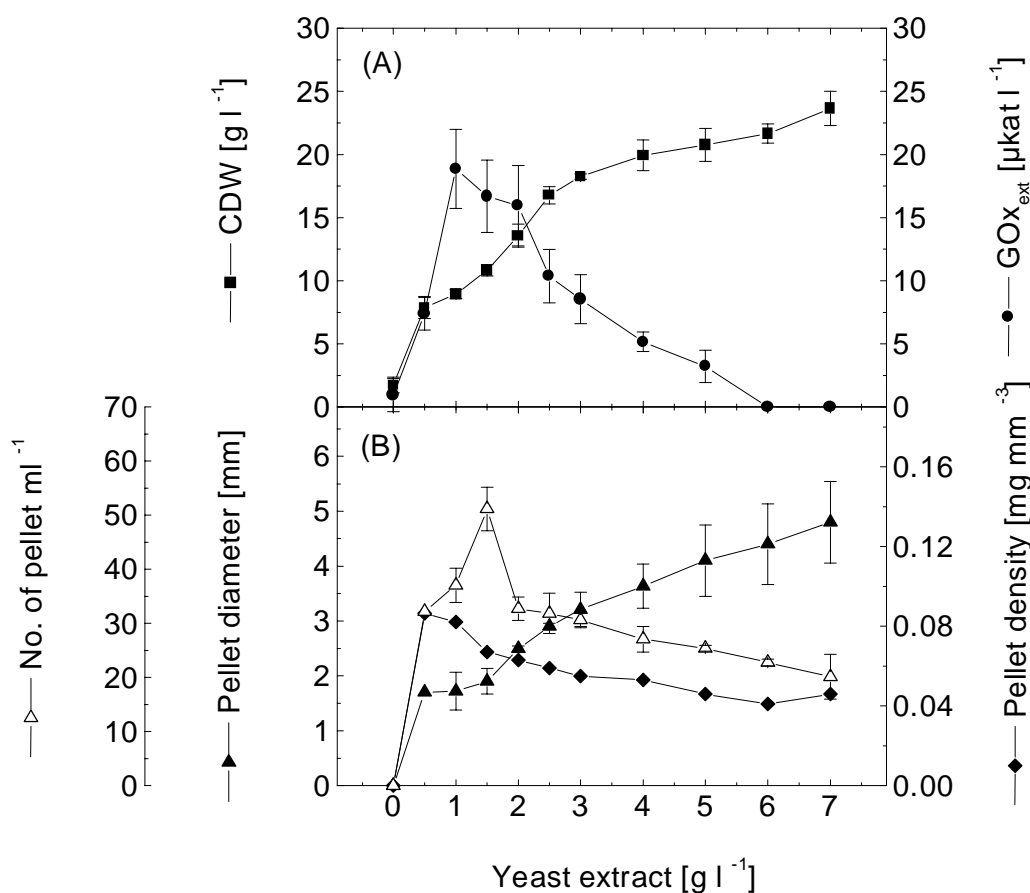
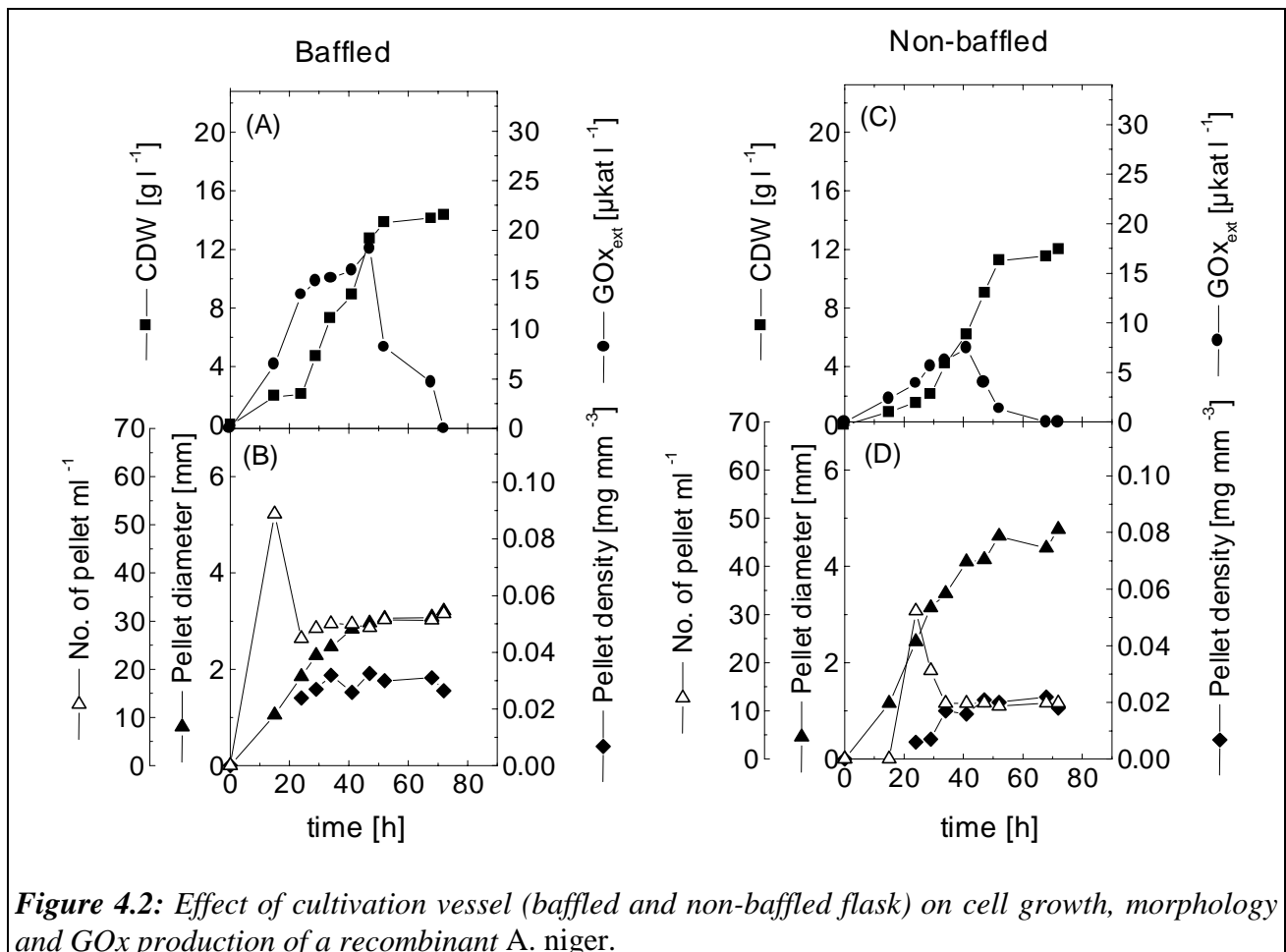


Figure 4.1: Effect of different concentrations of yeast extract on cell growth and GOx production in *A. niger* culture during shake flask cultivation.

4.1.1.2 Effect of the type of shake flask on growth and extracellular glucose oxidase production

To study the relation of extracellular GOx production to pellet properties in more detail, the recombinant fungus was grown on basic minimal medium supplemented with 2 g·l⁻¹ yeast extract in shake flasks with and without baffles [Fig. 4.2]. The presence of baffles is expected to result in higher shear stress causing the fungus to grow in pellets of smaller size. These experiments revealed that the final cell dry mass increased only by 20% while the maximum extracellular GOx activity increased by 140% when non-baffled shake flasks were replaced by shake flasks equipped with two baffles [Fig. 4.2].

An analysis of the fungal morphology in these two different types of flasks revealed more and smaller pellets per unit volume with an increased density in baffled compared to non-baffled shake flasks. These results clearly demonstrate that in shake flask cultures pellets of bigger size and lower density perform less efficiently with respect to the extracellular production of GOx.



Generally, during shake flask cultivations, a significant decrease in extracellular GOx activity was observed after 40 h. This drop in activity was due to the decrease in pH value caused by the transformation of glucose to gluconic acid.

4.1.1.3. Pellet morphology in shake flask culture

The typical pellet structure of *A. niger* in shake flask culture is represented in **Fig. [4.3]** and **Tab. [4.1]**. This pellet structure is characterized by four distinct layers with a hollow center.

The thin outer layer (Layer A) consists of a dense mycelial network. The adjacent and biggest layer (Layer B) shows a strong decrease in the mycelial density. The neighboring inner layer (Layer C) appears to be of intermediate density and is composed of hyphal cells and non-germinated spores. The next layer (Layer D) contains aggregates of non-germinated spores in addition to germinated spores with short hyphal tips. In large pellets (more than 2.5 - 3.0 mm in diameter) the inner core of the pellet appears hollow.

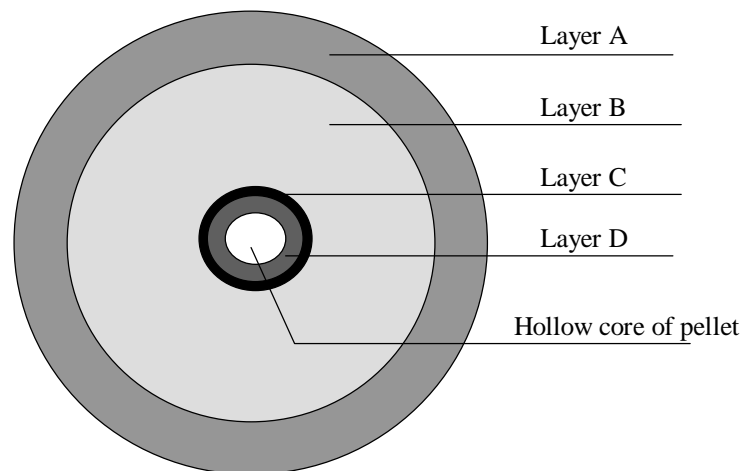


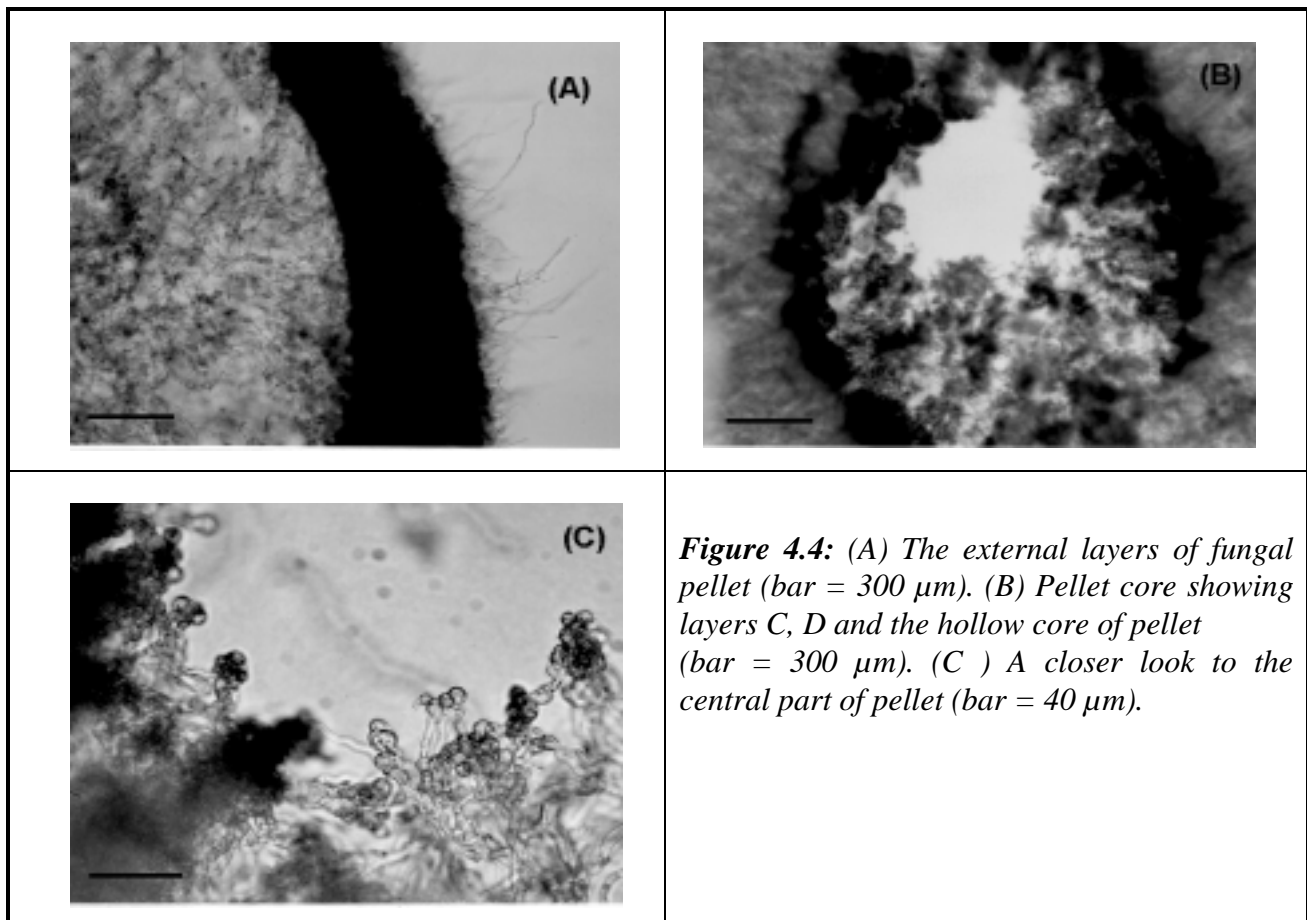
Figure 4.3: Schematic diagram of fungal pellet in shake flask after 48 h cultivation.

The structural analysis of fungal pellets obtained from shake flask cultures and the observation that pellets of larger size and lower density perform less efficiently, clearly demonstrate that the outer more dense layer exhibits the highest contribution to the extracellular production of GOx. The inner less dense layers are clearly subjected to substrate limiting conditions resulting in autolysis processes within the inner parts of pellet (Posser and Tough, 1991). The conclusion that the inner parts of the pellet is subjected to substrate limitation or even starvation conditions is additionally corroborated by the presence of fungal spores in the pellet core.

Table 4.1: Different zones inside the microbial pellet of *A. niger* NRRL 3 (GOD 3-18) after 48 h cultivation in shake flask.

Layer	Description
A	-This layer is composed of a highly dense mycelial net-work due to its direct exposure toward the medium and oxygen. This layer forms about 15-20% of pellet diameter. -This layer is the most physiologically active layer in the fungal pellet.
B	-This layer is composed of a less dense hyphal net-work compared to layer A. The reduction of growth may be due to the mass transfer limitation in the fungal pellet [Fig. 4.4A]. This layer forms about 50-55% of pellet diameter.
C	-This intermediate dense layer between B and D is composed of a combination between the hyphal cells from layer B and non germinated spores from layer D [Fig. 4.4B]. This layer forms about 2-4% of pellet diameter.
D	-This layer is composed of non-germinated spore aggregates and slightly germinated spores with a short germ tube [Fig. 4.4C] -This layer forms about 13-17% of pellet diameter.
E	-This hollow center of pellet forms about 5-10% of pellet diameter and is more predominant in large pellet.

Fig. 4.4 shows different layers in the fungal pellet after 48 h cultivation in non-baffled Erlenmeyer shake flask.



However, to abolish the above mentioned problems concerning the enzyme inactivation due to the drop of pH value and the large pellet formation, cultivation was done in 2-L bioreactor at constant pH 5.5.

4.1.2. Production of glucose oxidase in 2-L bioreactor

4.1.2.1. Spore aggregation and pellet formation during the early cultivation time

In order to study the kinetic of pellet formation as well as to optimize the growth conditions and enzyme production, *A. niger* NRRL 3(GOD 3-18) was cultivated in 2-L stirred tank bioreactor at constant pH value of 5.5. Agitation was performed by a single 4 bladed rushton turbine. At the beginning of the cultivation, the agitation was adjusted to 200 rpm for the first 5 hours, increased to 400 for 2 hours and again up to 600 rpm for the rest of cultivation time. The gradual increase in agitation speed was employed to inhibit spore floatation resulting in growth on the inner wall of the bioreactor as observed in cultivation started with high agitation speed (data not shown).

The number of spores at the beginning of cultivation time (t_0) was $1 \times 10^7 \text{ ml}^{-1}$. After 5 h, the number of free spores decreased to $0.21 \times 10^5 \text{ ml}^{-1}$ where the spore aggregates began to develop and reached $0.19 \times 10^5 \text{ ml}^{-1}$ with an average diameter of about $0.045 \pm 0.03 \text{ mm}$. Spore aggregates varied in shape and the number of spores per aggregate could not easily be determined. After 10 h, the number of free spores and aggregates continued to decrease. The decrease in aggregate number was due to the combination between aggregates after spore germination and subsequently the average diameter of aggregates increased to $0.15 \pm 0.05 \text{ mm}$. After 15 h of cultivation, no free spores were observed in the cultivation medium and the number of aggregates further decreased. After 20 h, a complete pellet structure with a number of $40 \pm 4 \text{ pellets ml}^{-1}$ and an average diameter of $1.3 \pm 0.2 \text{ mm}$ was formed [Tab. 4.2].

Table 4.2: The change in spore and bioparticle numbers and diameters during the early time of cultivation of *A. niger* in 2-L bioreactor.

Cultivation time [h]	No. of free spores per ml	No. of aggregates per ml	Average diameter of aggregates [mm]	Total number of bioparticles per ml
0	1.0×10^7	-	-	1.0×10^7
5	2.1×10^4	1.9×10^4	0.045 ± 0.03	4.0×10^4
10	3.0×10^3	1.0×10^3	0.15 ± 0.05	4.0×10^3
15	-	1.2×10^2	0.6 ± 0.2	1.2×10^2
20	-	40	1.3 ± 0.2	40 ± 4
30	-	40	1.3 ± 0.2	40 ± 4

4.1.2.2. Cell growth and glucose oxidase production and excretion

After the complete formation of compact pellet structure, both the pellet number per ml and diameter were constant at 40 pellets per ml and 1.3 mm during the rest of cultivation time. The increase in cell mass can be attributed to further cell growth inside the pellets as well as to a germination of spores in the pellet core. Concomitantly, the average pellet density increased from $4 \times 10^{-2} \text{ mg} \cdot \text{mm}^{-3}$ up to $9.8 \times 10^{-3} \text{ mg} \cdot \text{mm}^{-3}$ at the end of cultivation [Fig. 4.5B]. However, after 30 h glucose was converted completely to gluconic acid by the produced enzyme and the increase in CDW after this time was due to gluconic acid assimilation. The maximal cell dry weight reached 5 g l^{-1} after 61 h [Fig. 4.5A].

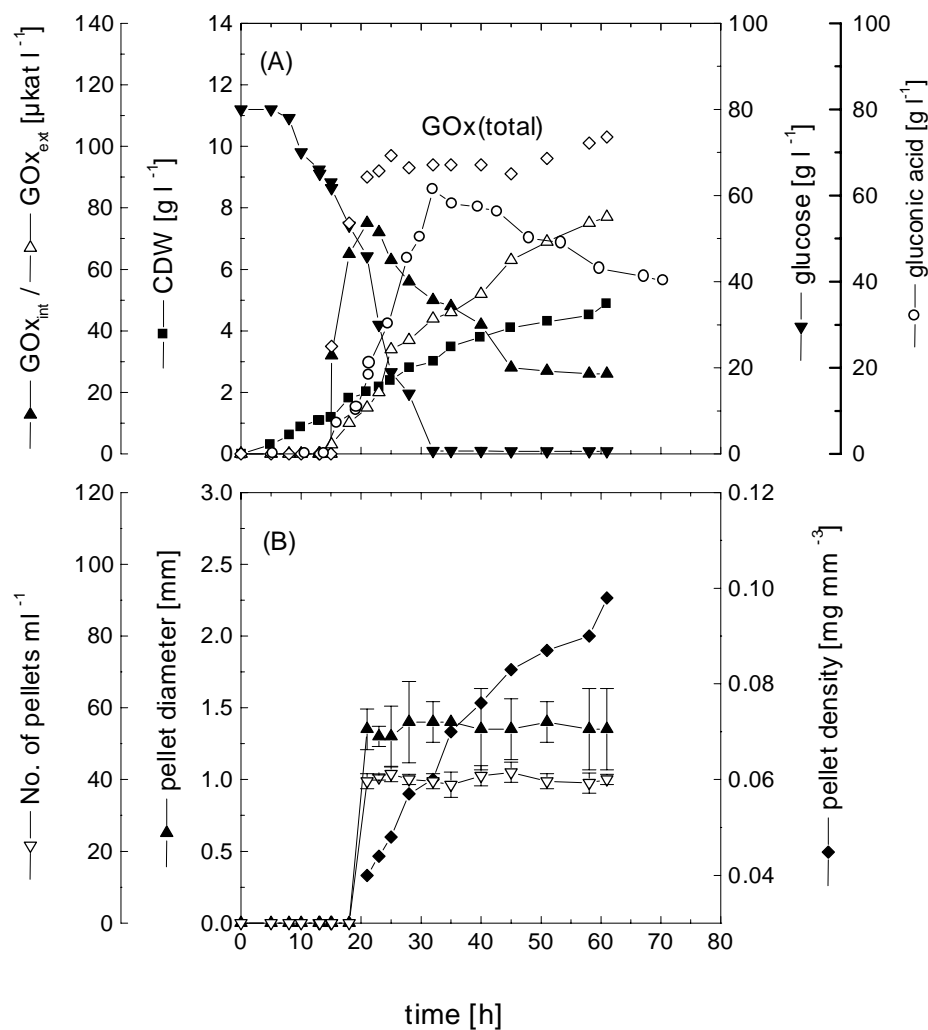


Figure 4.5: Cell growth and GOx production with a recombinant *A. niger* in 2-L bioreactor.

The maximal GOx produced (as a sum of extra- and intracellular content) was already observed after 20 h and remained constant for the rest of cultivation time. Therefore, the increasing extracellular GOx titer with cultivation time is mainly due to excretion of the enzyme rather than new production. After 61 h cultivation, the total GOx concentration was $103 \mu\text{kat l}^{-1}$ with only 75% of the enzyme excreted into the culture medium [Fig. 4.5A]. The average overall excretion rate of the enzyme, during the cultivation time from 20-61 h, was of about $1.97 \mu\text{kat h}^{-1}$.

The low enzyme production and excretion in this culture were due to morphological and physiological problems. It is known that the growth of fungal cells in pellet form larger than $400 \mu\text{m}$ in diameter causes severe mass transfer limitations between medium and pellet regarding substrate transport into the pellet as well as product transport from the pellet into the medium (Schügerl *et al.*, 1983). Moreover, not all cells were biologically active and the biologically active layer was restricted only in the outer layer of pellet. Also the conversion of glucose (a good carbon source for both cell growth and enzyme induction) to gluconic acid (a less suitable carbon source for cell growth and non-GOx inducer) due to enzyme production resulted in termination of enzyme synthesis. This problem was also observed in fed batch cultures feeded either with glucose or yeast extract. The growth morphology was identical in form of dense pellet in all cultures and the complete transformation of glucose to gluconic acid was observed after a few hours of enzyme production (data not shown). To overcome these problems, two strategies were applied to optimize the process:

- 1- Optimization of cell morphology through the production of smaller aggregates
- 2- Induction of GOx with non-glucose carbon source.

4.1.3. Production of glucose oxidase in 5-L bioreactor

In this experiment the production of GOx using glucose as a sole C-sources was studied in 5-L bioreactor agitated with three a 6-bladed rushton turbine. This type of bioreactor is characterized by a higher energy input per volume and better oxygen transfer rate compared to the previous type of bioreactor.

4.1.3.1. Effect of inoculum on growth morphology

The origin of inoculum showed a great influence on cell growth and GOx production. If spores were taken from stock glycerin culture (stored at -80°C) and plated one time on solid medium (CM-medium) and the spores produced were used directly as inoculum, the aggregation between spores will be less and the growth will be in a small aggregate form in a mycelial network. On the other hand, if the inoculum was obtained from a several time plated culture, the affinity between spores to aggregate increased under the same cultivation conditions, medium composition and inoculum size. In the first case where the inoculum was obtained from a multi-time plated cultures, the tendency of spores for aggregation was higher during the early time of cultivation and the morphology was in pellet form. The mechanism of pellet formation was followed in this experiment [Tab. 4.3].

Table 4.3: Spore aggregation and number of bioparticles during the batch cultivation of *A. niger* NRRL 3-(GOD3-18) in 5-L bioreactor using glucose.

Cultivation time [h]	No. of free spores [ml^{-1}]	No. of aggregate or pellets [ml^{-1}]	Total No. of bioparticles [ml^{-1}]
0	1.0×10^7	-	1.0×10^7
2	2.3×10^4	1.2×10^4	3.5×10^4
5	1.0×10^4	1.4×10^4	2.4×10^4
7.5	0.3×10^4	2456	5456
10	-	1543	1543
12.5	-	1084	1084
15	-	674	674
17.5	-	415	415
20	-	325	325
22.5	-	283	283
25	-	244	244
31	-	252	252
38	-	256	256
42	-	254	254
57	-	276	276
85	-	252	252
111	-	259	259
132	-	253	253

The aggregation between spores was carried out at the early phase and the variation in the number of spores forming one aggregate was observed. The number of both free spores and aggregates was decreased with time and after about 25 h all bioparticles were in pellet form. The pellet concentration reached about 250 ± 10 pellets ml^{-1} and kept constant during the rest of cultivation time. However, the pellet diameters were not equal and increased with time due to pellet growth [Fig. 4.6].

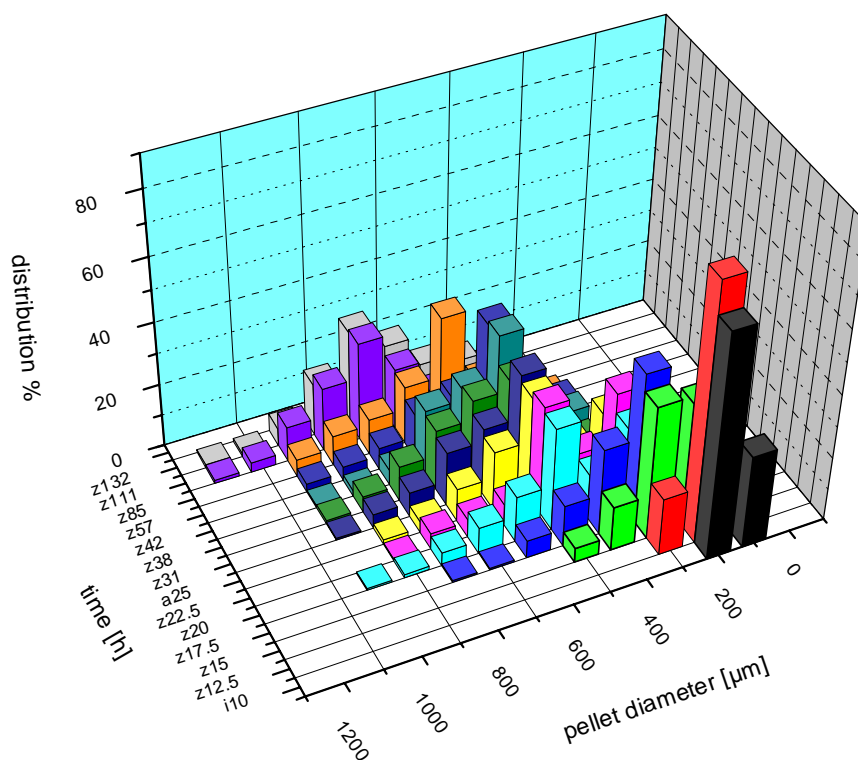


Figure 4.6: Change in pellet size distribution during batch cultivation of *A. niger* in 5-L bioreactor (glucose culture).

After 10 h, the pellet size distribution was narrow and the average of pellet diameter was between 100-200 μm . As the cultivation time increased, the pellet size distribution became wider with fractions between 400-1200 μm after 132 h [Fig. 4.6] and [Tab. 9.2.1, Appendix].

On the other hand, if the inoculum was in the form of spores obtained from a frozen glycerin cultures with only one time inoculation on an agar plate, the aggregation between the spores was less, and the spores formed a small micropellet in a mycelial network. Fig. 4.7 shows the morphological differences in both cultures during the cultivation time.

Culture inoculated by multiple plated spores / Culture inoculated by one time plated spores

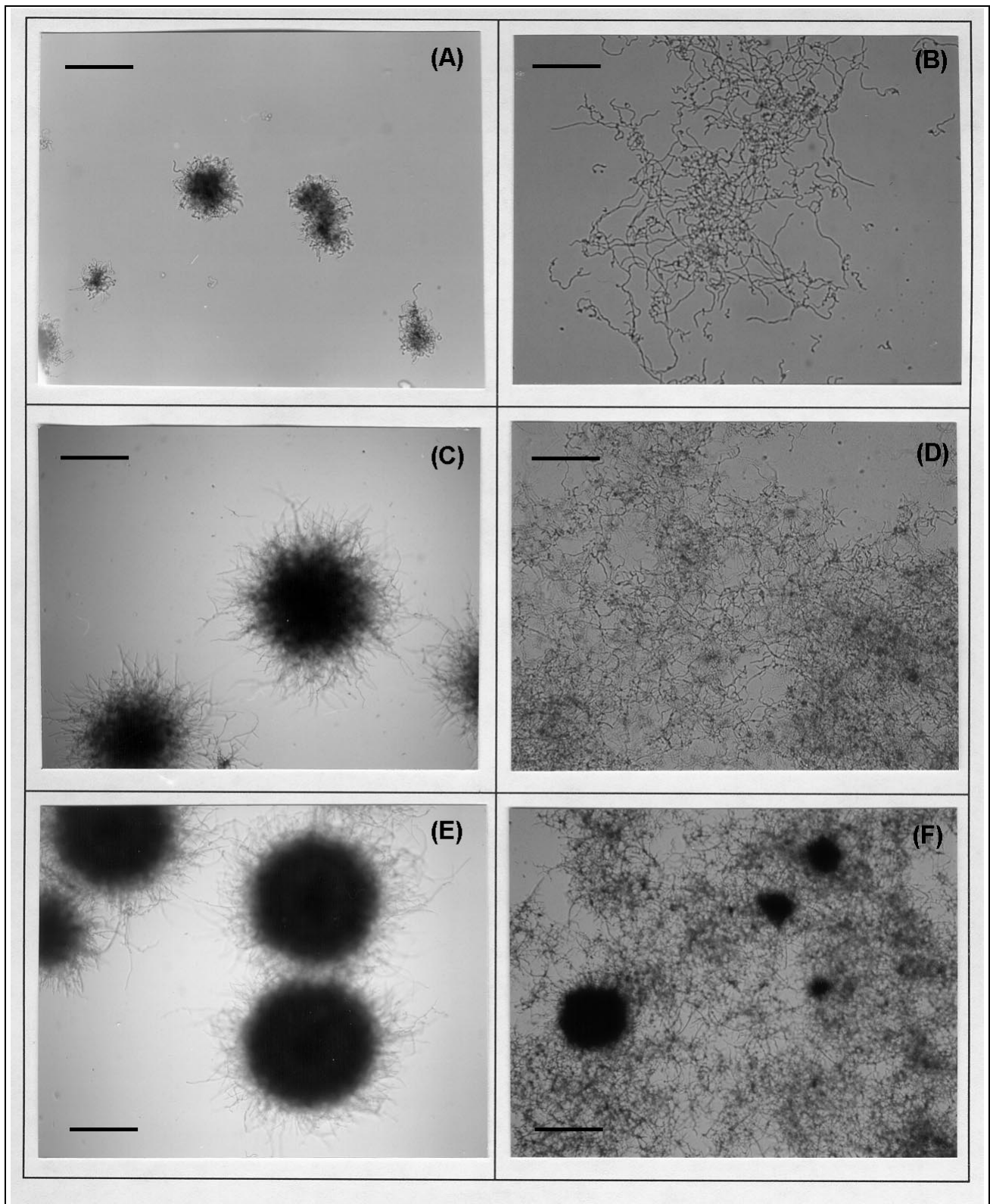
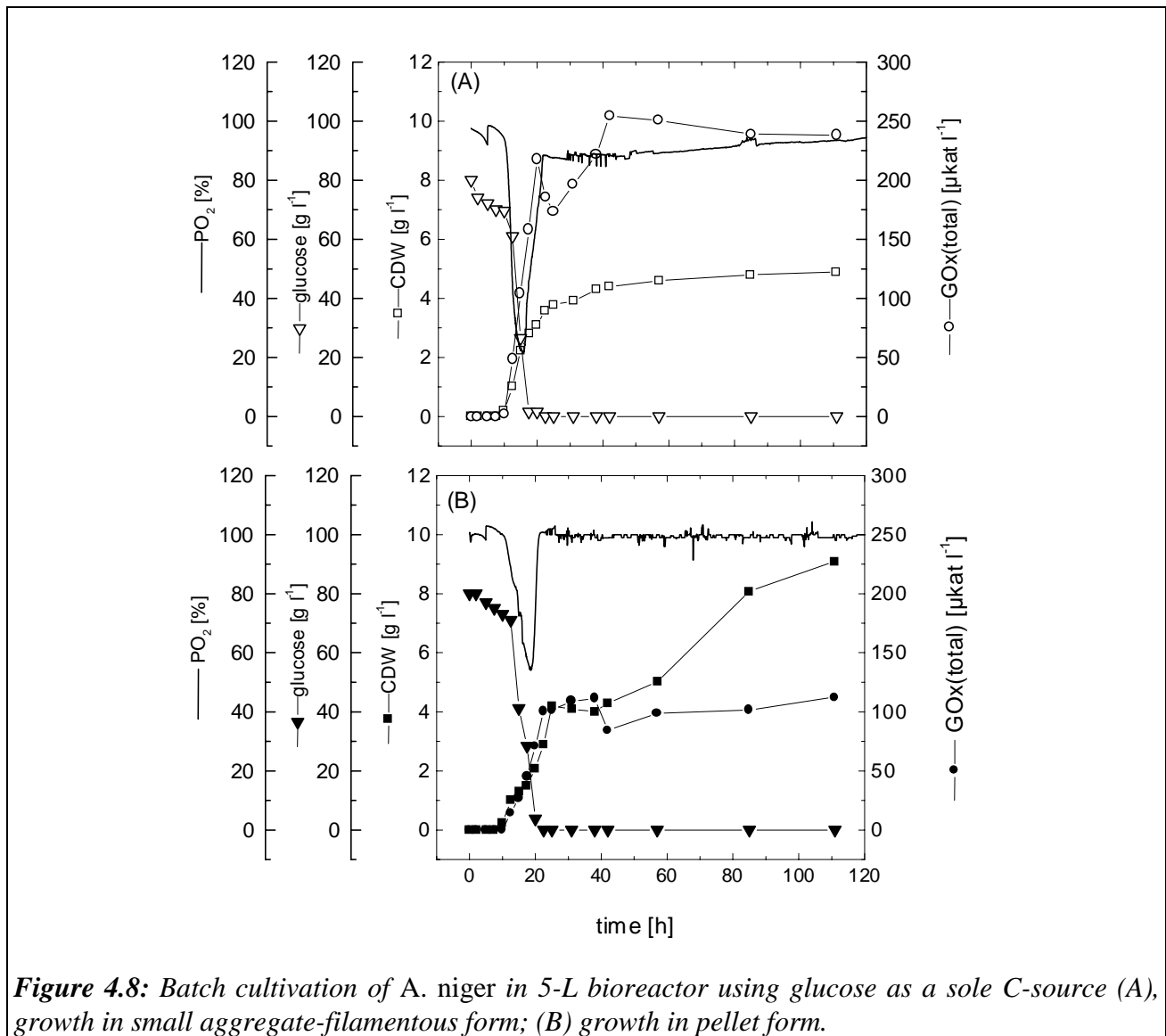


Figure 4.7: Morphological change of a recombinant *A. niger* in 5-L bioreactor in case of pelleted and filamentous growth in glucose cultures. (A, C and E for pelleted growth) and (B, D and F for filamentous growth) after 15 h, 25 h and 50 h, respectively. (bar = 300 μm).

4.1.3.2. Cell growth, glucose oxidase production and organic acid production

The difference in morphology caused significant differences in cell mass formation, substrate consumption and GOx production [Fig. 4.8] and [Tab. 9.1.1a, b, Appendix].



The growth started in both cultures after a lag phase of about 5 h and reached 3.6 and 2.9 g l⁻¹ CDW after 22.5 h for filamentous grown and pelleted grown cultures, respectively. After this time, the rate of increase in cell mass was less in both cultures due to the depletion of glucose, since glucose is converted to gluconic acid (a less suitable C-source for cell growth, Lakshminarayana *et al.*, 1969) by the produced enzyme. However, the rate of glucose oxidation to gluconic acid was different in

both cultures [Fig. 4.9]. The higher acid production rate in case of filamentous growth may be due to the higher rate of the enzyme production and excretion. Li and Chen (1994) found that the amount of gluconic acid produced by *A. niger* in submerged culture was closely related to the total amount of GOx produced. Träger *et al.* (1991) reported that the contribution of intracellular enzyme activity for gluconic acid production is about the half of the extracellular enzyme due to a diffusion limitation of substrates within fungal pellets. After 25 h, growth continued with a lower rate because of the assimilation of gluconic acid. After 111 h of cultivation, the cell mass produced in filamentous-grown culture was about 4.9 g l^{-1} (the half of pelleted growth). The reduced growth in filamentous-grown culture could be due to the cell destruction. It is well known that the sensitivity of cells to the hydrodynamic stress in filamentous-grown culture is higher compared to pelleted-grown one (Morimura *et al.*, 1992).

However, the gluconic acid was not only used for cell growth and respiration but also converted to oxalic acid. The utilization of gluconic acid in depletion of glucose was studied by Müller (1985, 1986) and it was found that *A. niger* can utilize gluconate as sole C-source and give rise mainly to oxalic acid and carbon dioxide. The rate of oxalic acid production in the filamentous-grown culture was higher than in pelleted one [Fig. 4.9]. The higher production rate of oxalic acid in case of a filamentous grown culture was due to the lower mass transfer limitation regarding substrate/product as discussed before in case of gluconic acid production.

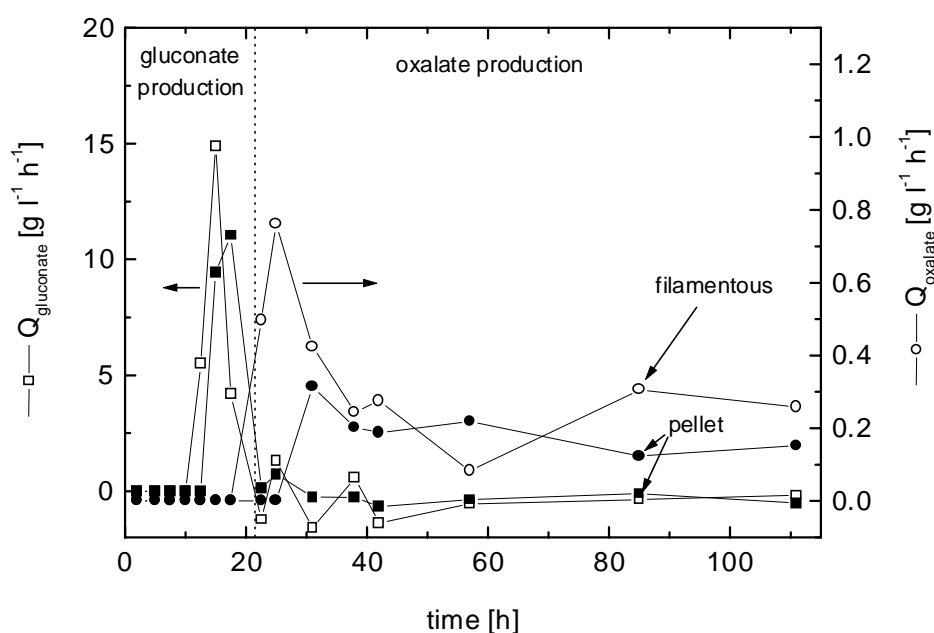


Figure 4.9: The rate of volumetric production of organic acids during GOx production and excretion by *A. niger*, open and close symbols represent growth in filamentous and pellet forms, respectively.

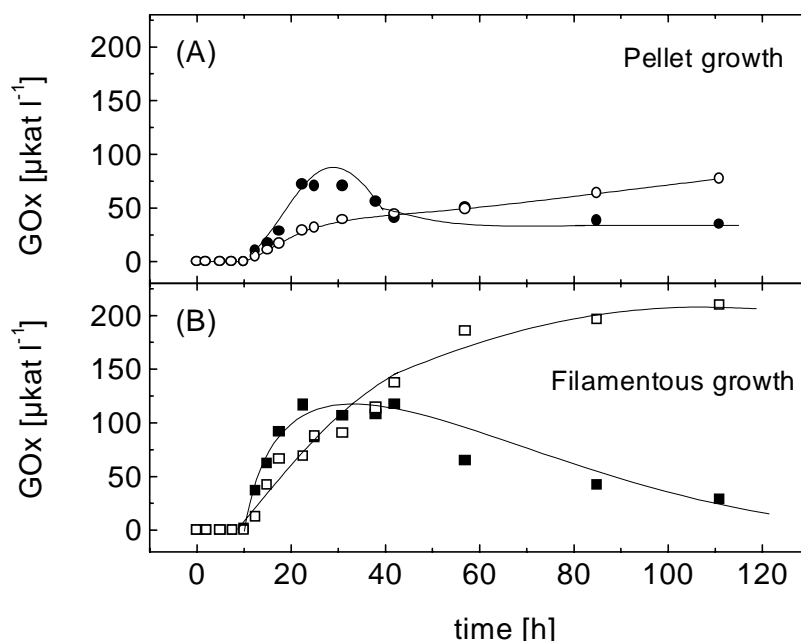


Figure 4.10: Distribution of GOx during cell cultivation in glucose cultures, closed and opened symbols represent intra- and extracellular enzyme, respectively.

In case of pelleted growth the maximal intracellular activity of $72 \mu\text{kat l}^{-1}$ was observed after 22 h and decreased with time due to enzyme excretion to the cultivation medium. At the end of cultivation 29.5% of the total GOx was still retained inside the cells. After 13 h, GOx was excreted into the cultivation medium, the extracellular concentration of GOx increased linearly with time until the end of cultivation. However, the maximal GOx production, as a sum of GOx_{int} and GOx_{ext} , of about $100 \pm 10 \mu\text{kat l}^{-1}$ was obtained after 22.5 h and kept more or less constant for the rest of cultivation time. At the time of maximal production, the cell dry weight was 4.45 g l^{-1} and increased again on the consumption of gluconate. Therefore, one may conclude that the newly formed cells on gluconate did not participate in any anabolic activities shared in GOx production. In case of mycelial grown culture the maximal GOx production, as a sum of GOx_{int} and GOx_{ext} , of about $220 \pm 20 \mu\text{kat l}^{-1}$ was obtained after 20 h of cultivation and kept constant for the rest of the cultivation. The maximal production of gluconic acid of 68.5 g l^{-1} was obtained after 20 h and decreased again due to its decomposition and re-utilization for oxalic acid production.

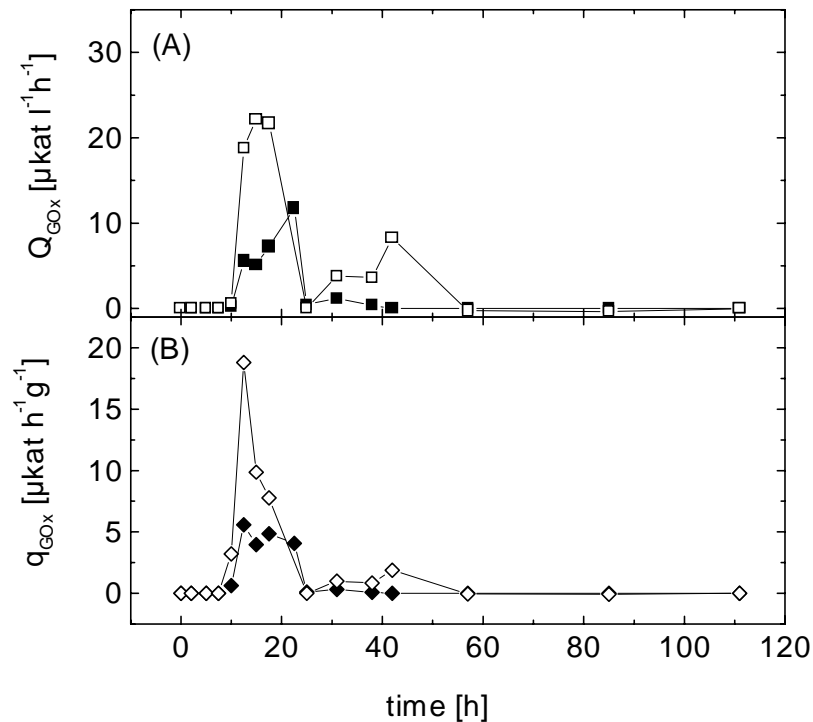


Figure 4.11: Volumetric and specific GOx production rates by *A. niger*, opened and closed symbols for filamentous and pelleted growth, respectively.

Since the type of spores used as inoculum in both cultures was the only varying factor and consequently the morphological change in both culture, it could be assumed that the morphology played a significant role in glucose oxidase production. However, not only the production of GOx was influenced by the morphology of growth but also the enzyme distribution was affected [Fig. 4.10]. The enzyme excretion to the cultivation medium was higher in case of filamentous grown culture. The lower excretion in pelleted grown culture may be due to mass transfer limitation within the pellet decreasing enzyme excretion to the cultivation medium.

A significant difference in both of volumetric and specific enzyme production rates between filamentous grown culture and pelleted one was also observed [Fig. 4.11]. The differences in cell biosynthetic capacity and excretion in both cultures were mainly due to the differences in oxygen supply to cells according to the morphological differences [Fig. 4.7].

It is well known that the biosynthesis of glucose oxidase by *A. niger* is limited due to insufficient oxygen transfer (Zetelaki, 1970) and especially extreme in case of pellets larger than 400 μm in diameter (Wittler *et al.*, 1986).

In addition, both oxygen consumption and carbon dioxide production rates were higher in filamentous grown culture compared to pelleted one during the enzyme production phase indicating a higher metabolic activity of the filamentous grown culture during this time [Fig. 4.12].

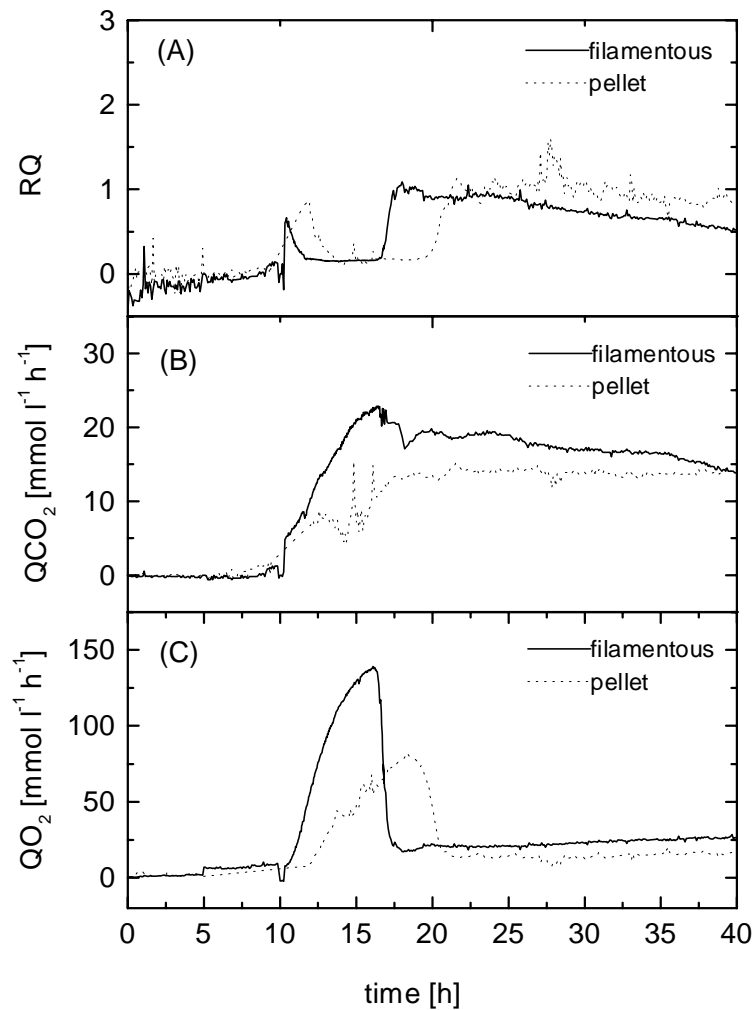


Figure 4.12: Effect of cell morphology on oxygen consumption and carbon dioxide production rates during cultivation of *A. niger*.

The difference between the oxygen consumption rate and the carbon dioxide production rate during the time of enzyme production may be due to the utilization of oxygen for both cell respiration and enzymatic conversion of glucose to gluconic acid in both cultures.

4.1.3.3. Carbon balances

According to a simplified carbon balance, only the carbon content of the cell mass, sugar consumed, organic acid produced and CO₂ evolved were considered. Both the share of yeast extract in medium and the share of the GOx in the carbon balance were neglected. The C-balance was determined according to the method of Nowakowska-Waszczyk and Sokolowski (1987), [Eq. 4.1].

$$X \cdot \sigma_x + Gn \cdot \sigma_{gn} + Ox \cdot \sigma_{ox} + dCO_2 \cdot \sigma_{CO_2} = S \cdot \sigma_s \quad [4.3]$$

The terms S , X , Gn , Ox and d are masses of utilized sugar and produced biomass, gluconic acid, oxalic acid and CO₂; their carbon content is denoted as σ_s , σ_x , σ_{gn} , σ_{ox} , σ_{CO_2} , respectively. The elemental analysis of the recombinant *Aspergillus niger* NRRL 3 (GOD 3-18) has the following composition CH_{1.74} O_{0.711} N_{0.117} (performed in Analytische Laboratorien Co. Gummersbach, Germany).

Figs. [4.13A and B] show the carbon balance in case of both filamentous-micropellet morphology and pellet morphology, respectively.

In case of filamentous growth form, the production of carbon dioxide was higher in spite of the lower biomass content compared to pelleted growth. This result gives also a direct indication of the influence of growth morphology on cell activity.

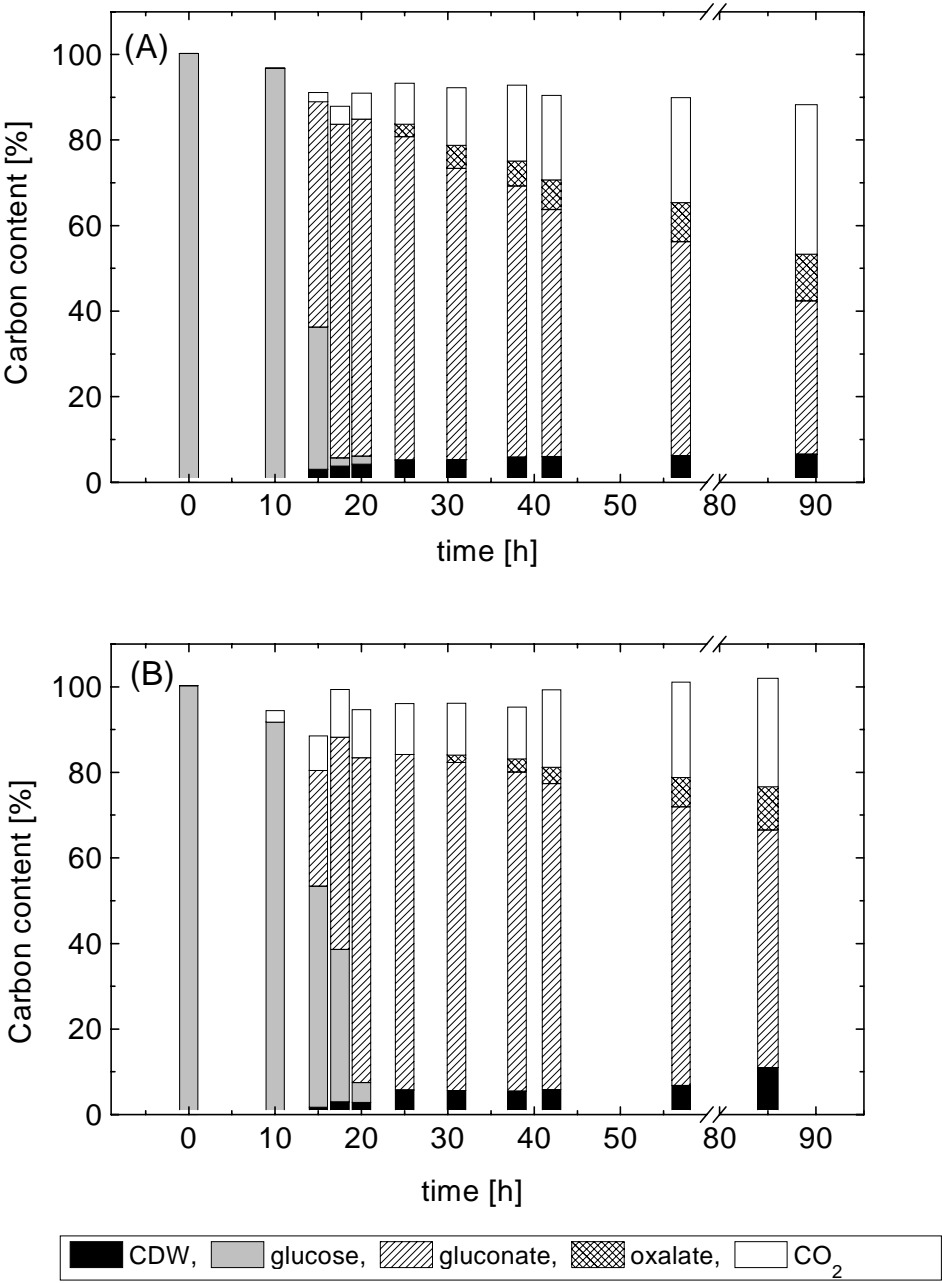


Figure 4.13: Carbon balances of *A. niger* during batch cultivation in glucose culture (A), growth in filamentous form, (B), growth in pellet form.

4.1.3.4. The relation between yield of enzyme production and growth morphology

As shown previously, the morphology of fungal growth plays a significant role in this process and an increase of about 2 times in enzyme production could be obtained only through the change of growth from pellet form to micropellet-filamentous form under the same cultivation conditions. A closer look to this process and calculation of yield coefficient ($Y_{P/X}$) as [$\mu\text{kat g}^{-1}$] shows that the specific yield of enzyme produced was more than two times higher in filamentous grown culture compared to pelleted one [Fig. 4.15].

The lower yield in case of pelleted grown culture was due to the composition of the biomass inside the pellet which can be divided into active and inactive fraction as described before. Assuming that the active growing layer of biomass is the only productive part, the yield of this biomass was more or less equal to the value of filamentous growth [Fig. 4.15]. The thickness of active grown layer (w) in this experiment was of about 200 μm . The calculation of active fraction of the cell dry weight was done as described previously in materials and methods [Eqs. 3.10-3.13] with the knowledge of diameters and distributions of pellets during the cultivation time [Fig. 4.6 and Tab. 9.2.1, Appendix]. The active fraction of biomass in pelleted grown culture is represented in Fig. 4.14.

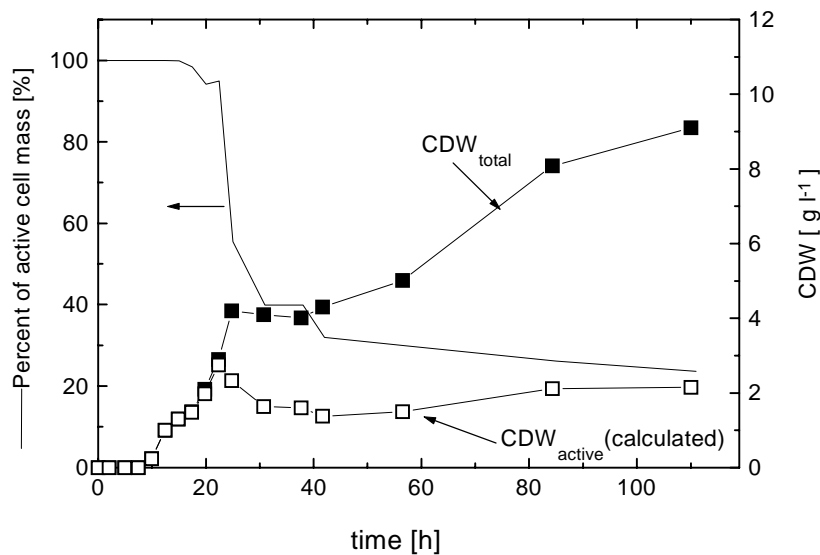


Figure 4.14: Percentage of active cell dry weight fraction during cell cultivation in pellet form in glucose culture.

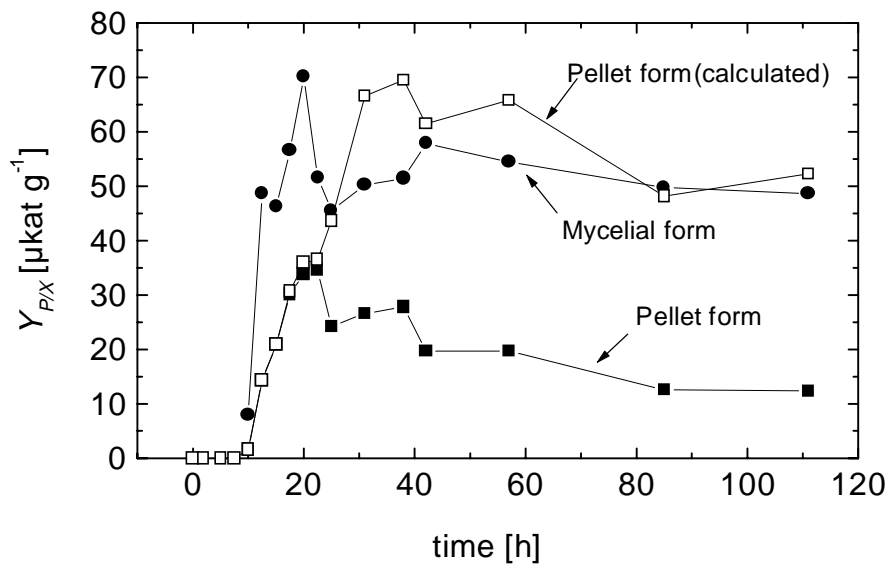


Figure 4.15: The yield of enzyme produced per cell dry weight during batch cultivation of *A. niger* in mycelial- and pellet form in glucose culture.

Summary

As shown in this chapter, three main factors influenced GOx production and excretion: pH of the cultivation medium, morphology of cell growth and conversion of substrate to less preferable form. With cultivation in a bioreactor at controlled pH we eliminated the pH effect which usually dropped to 2-3 due to gluconic acid production, resulted in a termination of enzyme production and inactivation of the produced enzyme as well. Also, using less hydrophobic spores (only one time transferred on agar medium) a decrease in aggregation between spores was achieved giving rise to the desired growth form (micropellet-filamentous form). Furthermore, a mathematical correlation between growth form and GOx production was established and the amount of enzyme produced in pelleted grown culture was calculated with the knowledge of pellet distribution, the thickness of actively grown cell layer and the yield of enzyme produced in a corresponding filamentous grown form under the same cultivation conditions.

4.2. Effect of different simple carbon sources on glucose oxidase production in shake flask cultures

In this experiment, a number of carbon sources were tested in order to determine their effect on cell growth and extracellular GOx production by recombinant *A. niger*. The C-sources were added to the minimal salt medium supplemented with 2 g l⁻¹ yeast extract with 2.7 mol l⁻¹ carbon (which is for example equivalent to 80 g l⁻¹ glucose). The results of the shake flask cultivations are represented in **Fig. 4.16**. After 48 h of cultivation, the maximal cell growth was obtained by using mannose as C-source followed by glucose and fructose. As the incubation time increased the cell mass increased and the final cell mass after 72 h was of the following order: fructose > mannose > glucose > xylose > arabinose > glycerol > ribose > galactose. In fact, the microorganism was able to grow on all carbon sources tested, but GOx was only produced in significant levels with xylose, mannose, glucose and fructose [**Fig. 4.17**]. With the exception of control (medium without C-source) which shows only a little number of very small pellets, the growth was mainly in pellet form with more or less the same diameter and number in all cultures under study. On the other hand, the pellet density varied from a loose pellet in case of glycerin culture to compact one in case of fructose culture.

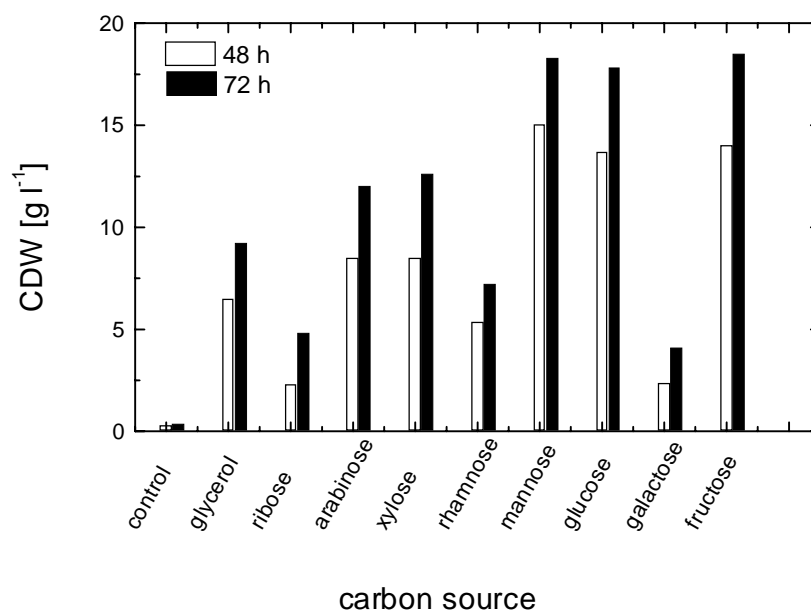


Figure 4.16: Effect of different mono-sugars on cell growth of *A. niger* in shake flask cultures (the control culture contains no carbohydrate).

After 48 h, the maximal extracellular production of GOx was obtained by using xylose as sole C-source and the amount of extracellular production of GOx was of the following order: xylose > mannose > glucose > fructose [Fig. 4.17]. Compared to glucose culture, GOx production was increased by about 121% and 47% in case of xylose and mannose cultures, respectively.

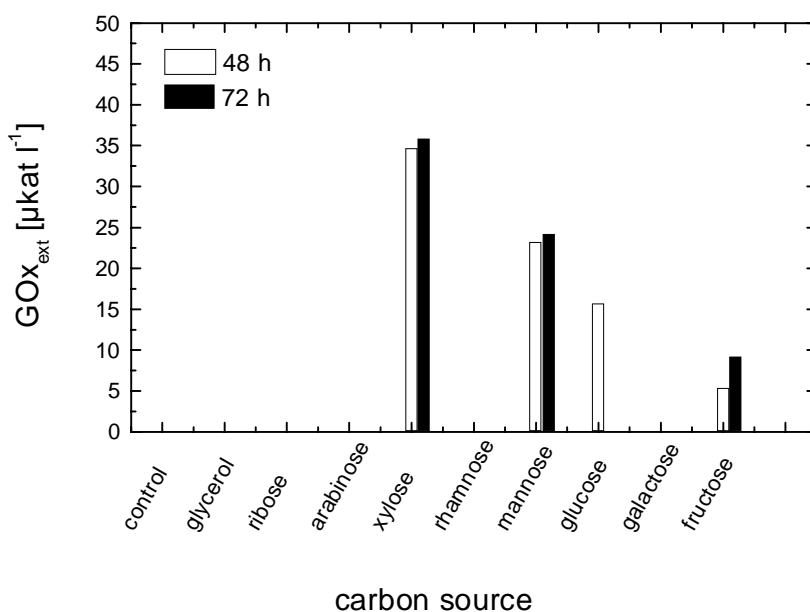


Figure 4.17: Effect of different mono-sugars on GOx production by *A. niger* in shake flask cultures.

The higher enzyme production in non-glucose cultures may be attributed to the pH of the medium. After 48 h of cultivation, the pH of glucose containing medium was dropped down to 3.35 whereas it was 4.75 and 4.68 in xylose and mannose containing media, respectively. The optimal pH for GOx production is between 5.5 and 6.5 (review of literature, this work) and the optimal pH of enzyme activity is 5.6 (Bentley, 1963). However, the obtained results showed no correlation between biomass production and enzyme activity. Petruccioli and Federici (1993) reported that among six different mono-sugar tested only glucose, fructose and mannose induce GOx production in *Penicillium variable* P 16 and xylose supports only cell growth without enzyme induction.

On the other hand, neither gluconic nor citric acid were produced upon using other C-source than glucose. However, the main advantage of the utilization of non-glucose sugars was the availability of the substrate for longer time without the conversion to other form by the produced enzyme. It is known that GOx has a high specificity to glucose compared to other carbohydrates (Rogalski, 1988; Schomburg and Stephan, 1995).

To confirm the result of GOx induction using fructose and xylose in this strain as well as for further optimization of this process, cell growth and enzyme production were further studied in a 5-L bioreactor.

4.3. Production of glucose oxidase using fructose in 5-L bioreactor

4.3.1. Cell growth, glucose oxidase production, substrate consumption and organic acid production

The suitability of fructose for cell growth and GOx production and excretion was studied in basal medium supplemented with 2 g l⁻¹ yeast extract. The inoculum was in form of 1×10⁷ spores ml⁻¹ obtained from a densely conidiating culture grown on CM agar medium, previously inoculated with a spore suspension stored in a frozen glycerin solution at -80°C for about 48-72 h. The agitation speed was 200 rpm for the first 5 h and raised up to 800 rpm for the rest of cultivation time.

After 5 h of cultivation spore aggregates were observed in the cultivation medium without germination. The first rise of germ tube was observed after 10 h of cultivation. Five hours later extensive growth in the form of small pellets with radial hyphal growth was observed. Cell growth increased exponentially and reached a maximal cell concentration of 11.5 g l⁻¹ after 30 h which remained more or less constant for the rest of cultivation [Fig. 4.18b]. During the exponential growth phase the yield of cell produced per substrate consumed [$Y_{x/s}$] was 0.36 g g⁻¹. After that time, fructose was mainly utilized for cell maintenance, enzyme production and synthesis of oxalic acid [Fig. 4.18a] and [Tab. 9.1.2, Appendix] and not for further formation of new cell mass. The consumption of sodium nitrate began according to cell growth after 15 h and after 51 h of cultivation about 90% of the initial concentration was consumed.

Glucose oxidase production started after 15 h and reached a maximal value of 180 µkat l⁻¹ after 40 h (calculated as a sum of extra- and intracellular activity) and was more or less constant for the rest of the cultivation time [Fig. 4.18c]. The maximal volumetric production rate of GOx [Q_{GOx}] was observed after 15 h (a time of extensive cell growth) and reached about 20 µkat l⁻¹ h⁻¹ [Fig. 4.19b]. On the other hand, the specific enzyme production rate [q_{GOx}] showed also a maximal value of about 5 µkat h⁻¹ g⁻¹ at 20 h [Fig. 4.19a].

About 85% of the produced enzyme at 40 h (the time of maximal enzyme concentration) was localized inside the cell and only a small fraction was excreted into the cultivation medium. The excretion rate of enzyme gradually increased with time but at the end of cultivation more than 50% of GOx were still localized inside the cell. The decrease in enzyme excretion compared to glucose culture was due to the morphological structure of fungal growth. In case of the cultivation with fructose growth was mainly in large and dense pellet structure.

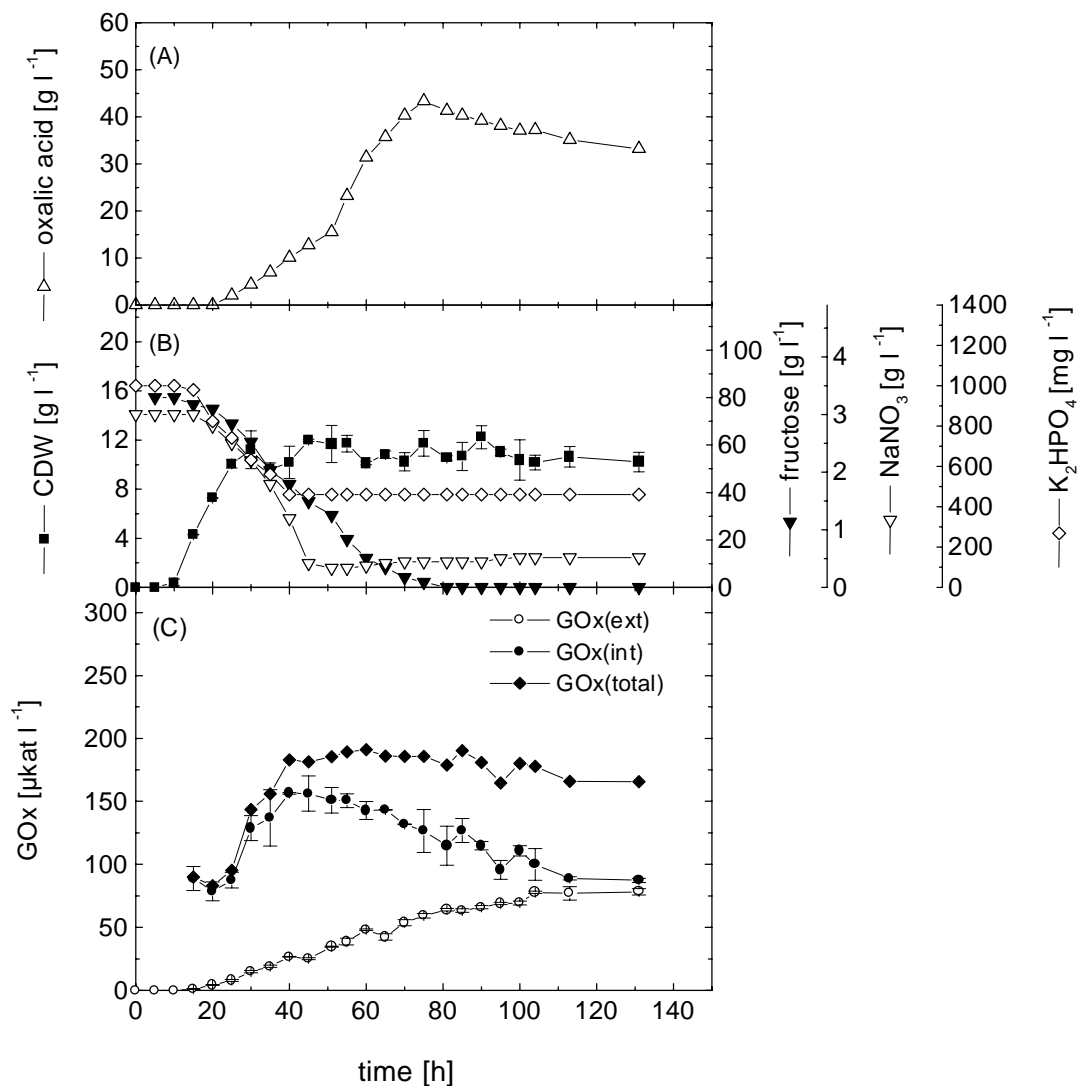


Figure 4.18: Growth curve of *A. niger* in 5-L bioreactor using fructose as a sole C-source.

During cultivation, sodium hydroxide (2.5 mol l⁻¹) was used to neutralize the acidity of medium and to keep the pH at 5.5 (the most suitable value for GOx synthesis).

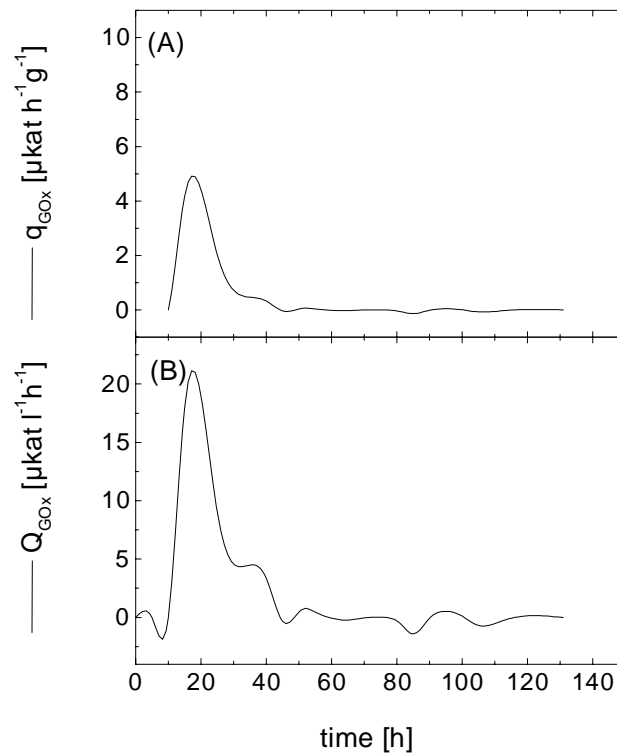


Figure 4.19: Volumetric and specific GOx production rates during a recombinant *A. niger* cultivation in fructose culture.

The consumption rate of sodium hydroxide was proportional to the rate of oxalic acid synthesis. The production of oxalic acid started after 20 h and increased with the time reaching a maximal volumetric production of about 43 g l⁻¹ after 75 h. The maximal rate of oxalic acid production of about 2 g l⁻¹ h⁻¹ was observed after 60 h [Fig. 4.20].

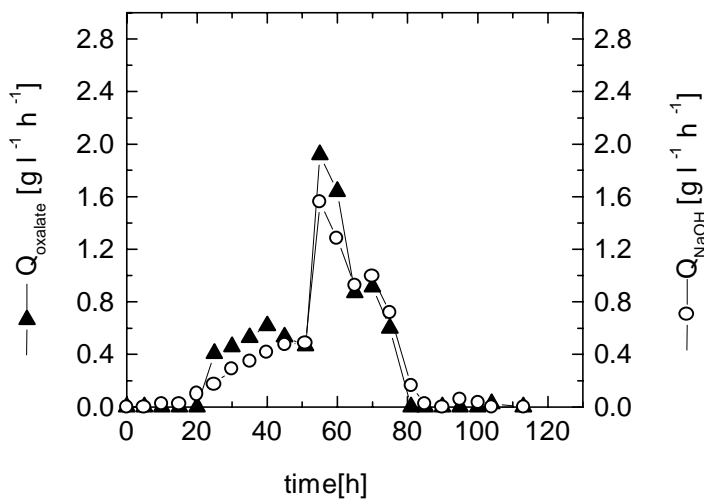


Figure 4.20: Oxalate production rate and sodium hydroxide consumption rate during submerged cultivation of *A. niger* in fructose culture.

4.3.2. Carbon balance

The carbon balance was also studied in fructose culture and calculated as described in the previous experiment as follows:

$$X \cdot \sigma_x + Ox \cdot \sigma_{ox} + dCO_2 \cdot \sigma_{CO_2} = S \cdot \sigma_s \quad [4.4]$$

The terms S , X , Ox and d are masses of utilized sugar, produced biomass, oxalic acid and CO_2 ; their carbon contents are denoted as σ_s , σ_x , σ_{ox} , σ_{CO_2} , respectively.

The results in **Fig. 4.21** show that the production of carbon dioxide in this culture was higher compared to the glucose culture either grown in pellet or filamentous form. Also the utilization of fructose for respiration and oxalic acid production was more efficient than gluconic acid.

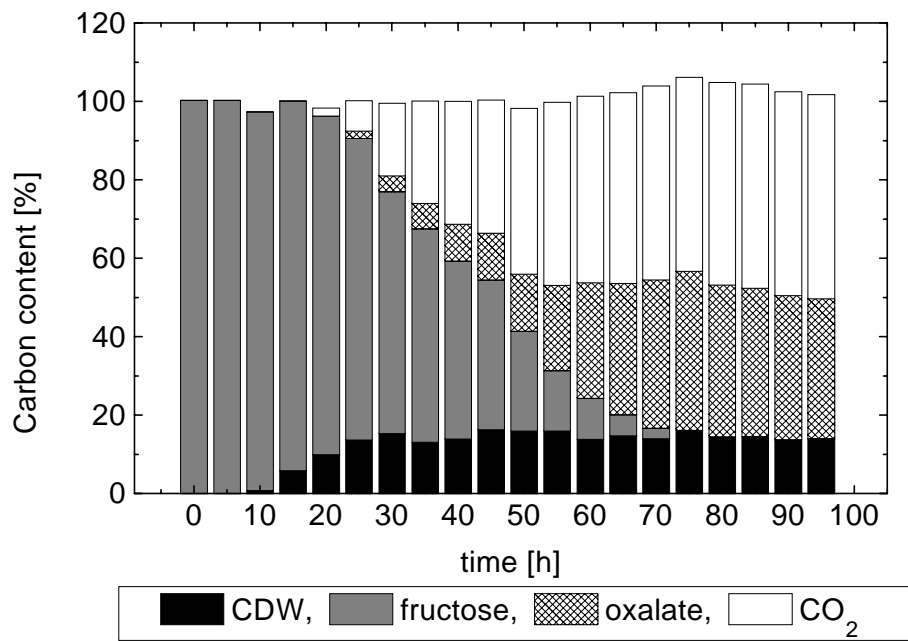


Figure 4.21: Carbon balance during cell cultivation in fructose culture.

The induction of GOx in cultivation with fructose as a sole C-source was studied before by different investigators. Witteveen *et al.* (1990) studied the induction of GOx in different mutant strains of *A. niger* using fructose. They found that the amount of GOx produced in fructose culture was only 3% compared to glucose culture with wild type. After cell mutation with UV technique the produced mutants showed different responses for GOx induction by fructose and reached sometimes higher titer than the glucose culture. In another studies using a wild type *A. niger*, the fructose supported only cell growth and the amount of enzyme produced was about 8% of glucose culture

(Hatzinikolaou and Macris, 1995). In another producer strain such as *Penicillium variabile* fructose supports also cell growth and the GOx produced was about 82% of glucose culture (Petruccioli and Federici, 1993). Recently Rando *et al.* (1997) studied the induction of GOx in a newly isolated strain of *Penicillium pinophilum* using fructose as C-source. They reported that the production of GOx increased up to 70 folds compared to glucose culture under the same cultivation conditions. These results indicate that the induction of GOx with fructose is highly strain specific. However, most of these studies were done in shake flask and no details about cell morphology were given. From our results presented above we conclude that fructose supports cell growth better than glucose in a recombinant *A. niger* (GOD 3-18). On the other hand, the production of GOx was less induced compared to the glucose culture.

4.4. Production of glucose oxidase using xylose in 5-L bioreactor

4.4.1. Cell growth, glucose oxidase production, substrate consumption and acid production

In this experiment xylose with a concentration of 80 g l^{-1} was used as a sole C-source in a basal medium supplemented with 2 g l^{-1} yeast extract. Inoculum preparation and cultivation conditions were carried out as described in the previous experiments.

After a lag time of about 6 h, spores germinated and the growth was more extensive than glucose or fructose cultures. The maximal cell mass was obtained after 40 h reaching 16.3 g l^{-1} and kept constant for the rest of cultivation time. The consumption of xylose started after about 15 h and increased with the time until cultivation was terminated. However, during exponential growth the yield of biomass produced per carbon source consumed [$Y_{x/s}$] was 0.445 g g^{-1} . The consumption of sodium nitrate started after 20 h and after 45 h 85% of the initial concentration was consumed. The delayed nitrate consumption may be due to the presence of yeast extract in the medium (a preferable N-source compared to nitrate).

Fig. 4.22 and **Tab. 9.1.2** [Appendix] show the cell growth, substrate consumption, enzyme production and different metabolites produced during batch cultivation in xylose culture. The intracellular GOx also increased with the time up to 35 h and then decreased due to the enzyme excretion to the cultivation medium. The maximal GOx production, as a sum of GOx_{int} and GOx_{ext} , of about $650 \mu\text{kat l}^{-1}$ was obtained after 40 h of cultivation. However, as shown in **Fig. 4.22** the first rise of GOx in the cultivation medium was observed after 20 h and increased gradually with the time.

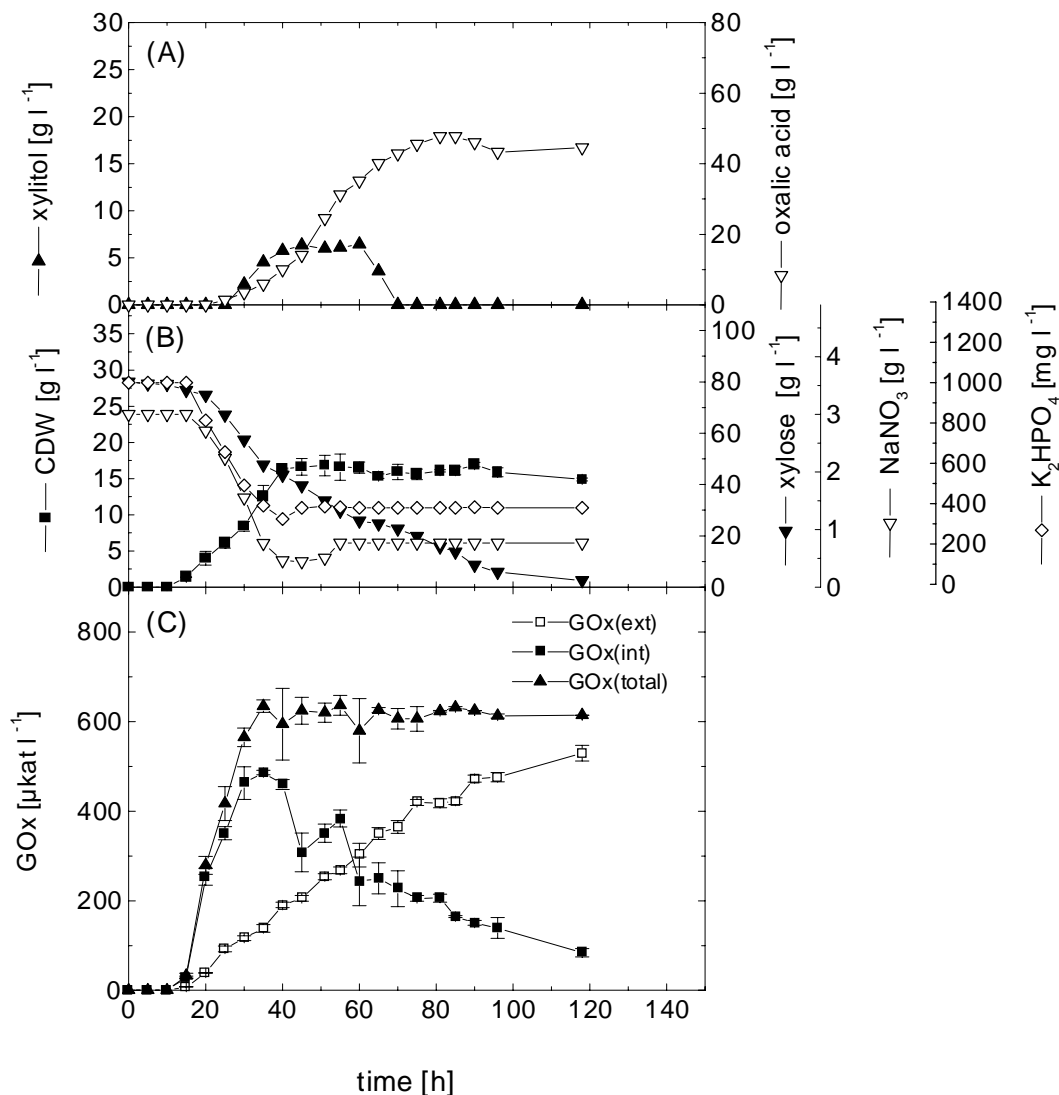


Figure 4.22: Growth curve of *A. niger* in 5-L bioreactor using xylose as a sole C-source.

The values of both volumetric and specific enzyme production rates show a maxima of about $50 \mu\text{kat h}^{-1}$ and $12 \mu\text{kat g}^{-1} \text{h}^{-1}$, respectively, at 20 h and then decreased gradually with time reaching to zero after 40 h. The longer production phase of GOx compared to the glucose culture was due to further cell growth and due to the availability of the substrate, xylose, for longer time before its transformation to oxalic acid.

Xylitol was formed in this culture as an intermediate product. The first detection of xylitol was after about 30 h of cultivation reached a maximum of 6.3 g l^{-1} after 40 h and decreased again either due to consumption or transformation to oxalic acid. After 70 h no xylitol was determined in the cultivation medium.

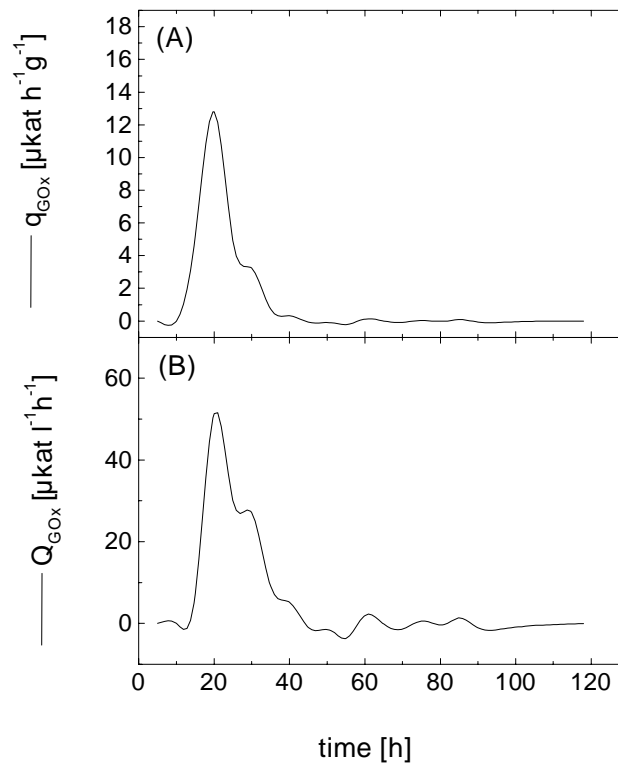


Figure 4.23: Volumetric and specific GOx production rates during cell growth in xylose culture.

It is also interesting to note that neither gluconic acid nor citric acid were produced during this cultivation. On the other hand, sodium hydroxide solution of 2.5 mol l^{-1} was added continuously to neutralize the acidity of oxalic acid [Fig. 4.24]. The concentration of oxalic acid reached 44.5 g l^{-1} after 118 h of cultivation [Fig. 4.22a].

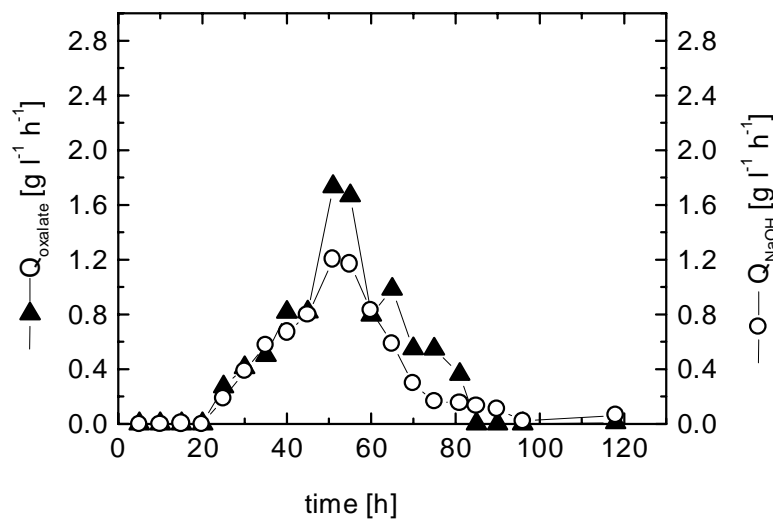


Figure 4.24: Oxalate production rate and sodium hydroxide consumption rate with the function of time.

4.4.2. Carbon balance

The calculation of carbon balance was done during cultivation time according to the following equation:

$$X \cdot \sigma_x + Ox \cdot \sigma_{ox} + Xy \cdot \sigma_{xy} + dCO_2 \cdot \sigma_{CO_2} = S \cdot \sigma_s \quad [4.5]$$

The terms S , X , Ox , xy and d are masses of utilized sugar, produced biomass, oxalic acid, xylitol and CO_2 ; their carbon contents are denoted as σ_s , σ_x , σ_{ox} , σ_{xy} , σ_{CO_2} .

As shown in **Fig. 4.25**, xylose was converted to biomass, xylitol, oxalic acid and carbon dioxide. After 40 h of cultivation the biomass represented about 20% of total carbon in this culture and remained constant in this percent for the rest of cultivation. As the cultivation time increased the xylose converted mainly to organic acid and carbon dioxide. At the end of cultivation about 35% of the initial carbon from xylose was converted to carbon dioxide, 37% converted to oxalic acid and only about 3% of carbon remained in the initial form (xylose).

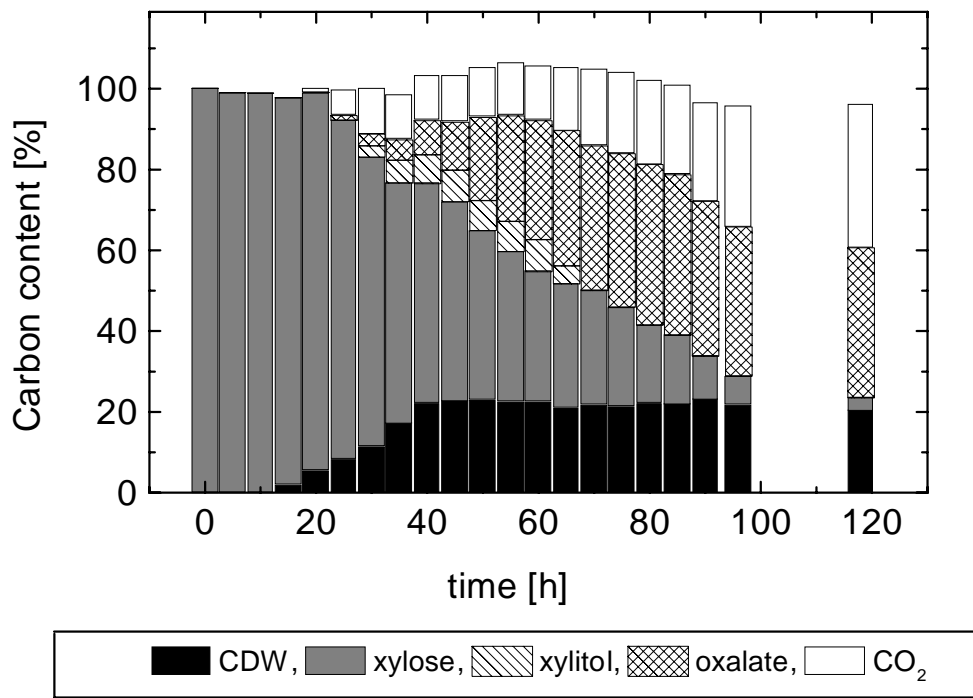


Figure 4.25: Carbon balance during cell cultivation in xylose culture.

4.5. Summary of cultivations using different C-sources in 5-L bioreactor

4.5.1 Effect of different C-sources on cell growth and glucose oxidase production and excretion

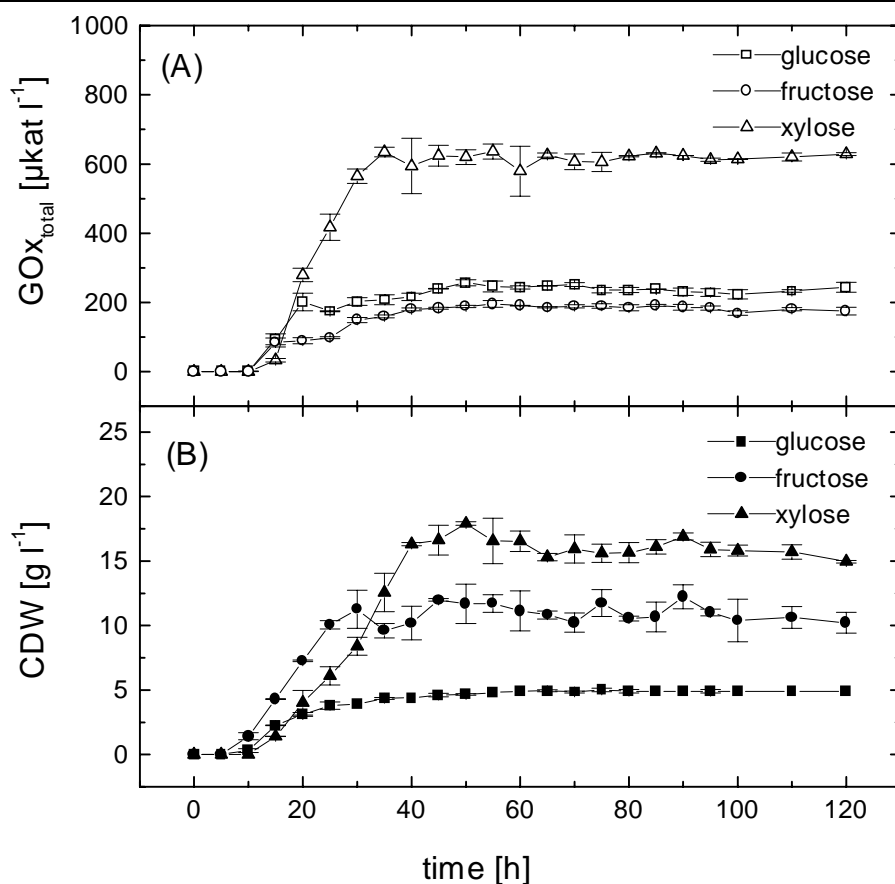


Figure 4.26: Effect of different carbon sources on cell growth and total volumetric GOx production (as a sum of cell-associated and extracellular enzyme) in stirred tank bioreactor batch culture. Data were taken from two different cultivations carried out under identical conditions. The inocula used in these experiments were of the same treatment (they came from a one time transfer on agar plate to minimize spore aggregation, see glucose experiment).

The different carbon sources supported cell growth and GOx production with different extent [Fig. 4.26]. The higher cell growth was observed in xylose culture followed by fructose and glucose. On the other hand, the production of GOx in xylose culture was more than 2-fold higher compared to glucose culture. The decrease in GOx production in fructose culture was mainly due to the growth in large compact pellet form (independently of the inoculum used). To eliminate the dependence of GOx production on cell concentration, a plot of specific enzyme activity

versus cultivation time is presented in **Fig. 4.27**. The specific yield of enzyme production was of the following order glucose > xylose > fructose

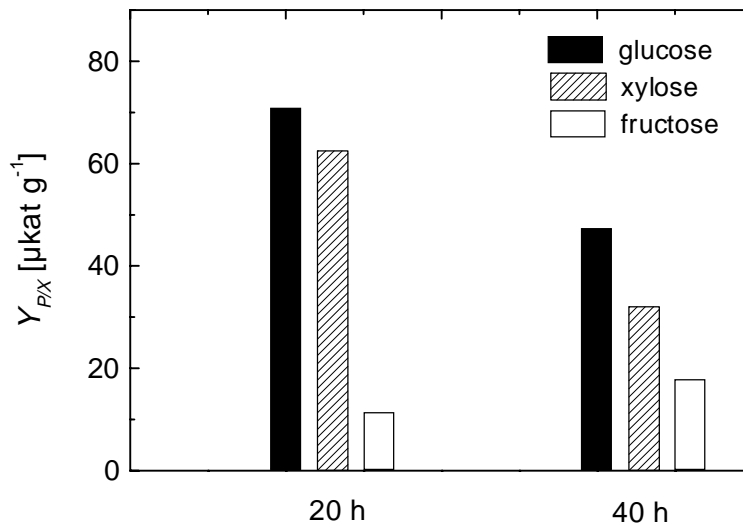


Figure 4.27: The amount of enzyme produced per unit biomass after 20 h (the time at which the maximal enzyme produced in glucose culture was observed) and 40 h (the time of maximal enzyme production in xylose and fructose culture).

However, not only the yield of enzyme was influenced by the C-source but also the excretion of the produced enzyme was highly affected. The intracellular/extracellular ratio of GOx varied with the type of C-source used. As shown in **Fig. 4.28** the time at which 50% of the enzyme excreted to the cultivation medium (T_{50}) was dependent on the C-source applied.

In case of glucose culture T_{50} was 31 h (11 h after reaching a maximal GOx production). In case of xylose culture T_{50} was 54 h (about 14 h after reaching a maximal GOx production). In case of fructose culture T_{50} was nearly reached after 120 hours.

The differences in enzyme excretion between glucose, xylose and fructose were mainly attributed to cell morphology which was in filamentous-micropellet form in the former two cases and in compact pellet form in fructose culture.

Therefore, we conclude that the rate of enzyme excretion is mainly dependent on cell morphology in spite of the C-source applied.

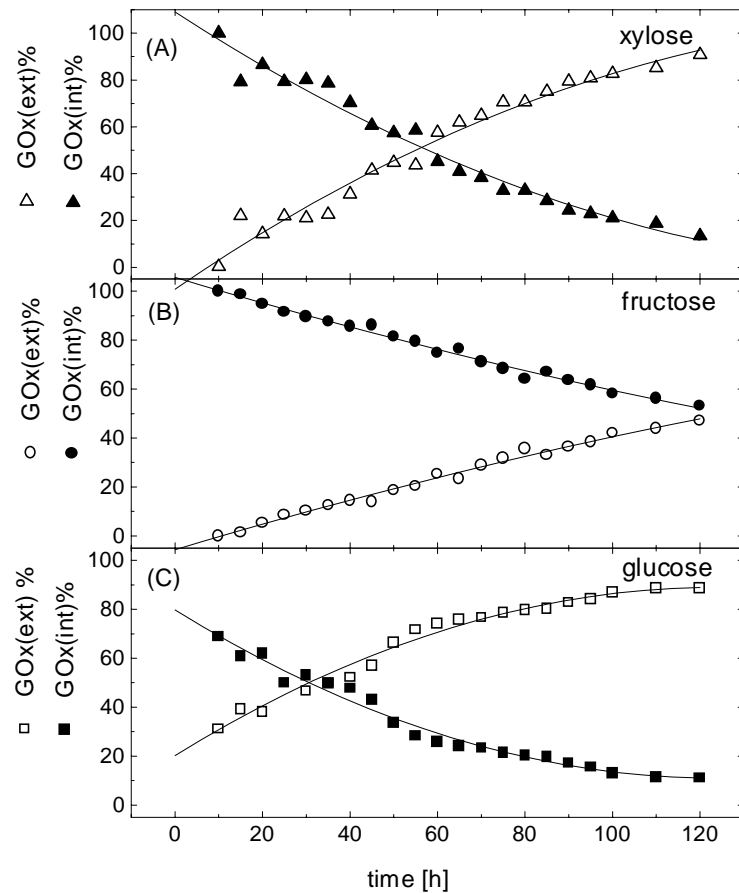


Figure 4.28: The distribution of GOx activity in both cell-associated and extracellular pools with the function of carbon source used.

4.5.2 Spore aggregation and growth morphology

Because pellet formation in *A. niger* is of aggregative type (Metz and Kossen, 1977) the interaction between spores during the early time of cultivation in submerged culture is the critical step for determination of fungal growth morphology.

After inoculation of medium with spores, two forces are working against each other: the aggregation force between spores and the dispersion force caused by agitation. The aggregation force mainly depends on the spore hydrophobicity which is very high in case of aerial spores grown in surface culture (Muñoz *et al.*, 1995). This hydrophobicity is due to a specific hydrophobic protein (Wessels, 1997). On the other hand, the dispersion force which is dependent on the impeller speed works against the aggregation force (Mitard and Riba, 1988). A high decrease of spores hydrophobicity occurs usually during the spore swelling and they become wettable.

Therefore, it can be concluded that if the swelling time is high the probability of aggregation between spores is more pronounced.

Cultivation in glucose culture

In case of growth in micropellet-mycelial form in glucose culture we presume that in glycerin culture the spores were wettable due to the dispersion in glycerin solution. After sub-cultivation on surface culture the induction of hydrophobic protein of the outer layer of spore was re-induced. As the sub-culturing progressed, the extensive hydrophobicity of spore recovered as well. Thus, as the hydrophobicity increased the probability of spore aggregation and pellet formation increased concomitantly.

Cultivation in fructose culture

In case of fructose the spore swelling and germination were delayed compared to glucose and xylose cultures (inoculated from the same type of inoculum). This resulted in maintenance of the spores hydrophobicity for a longer time, increasing the affinity of aggregation between spores. Thus, the growth was mainly in form of large pellets in this culture.

However, it is worthy to note that the spores produced from submerged culture (in the case of fructose culture) were neither germinated nor aggregated and remained in a single free form until the end of cultivation time. It is known that the spores formed in submerged culture are hydrophilic (Muñoz *et al.*, 1995). This resulted in a lower aggregation force between spores.

4.5.3 Oxalic acid production

In all cultures under study, oxalic acid accumulated at high concentration [Fig. 4.29]. The production of oxalic acid carried out either directly from the sugar used (in case of fructose and xylose) or through the decomposition of gluconic acid (in case of glucose culture). In the former case, the produced oxalic acid was more than 3 times higher compared to the production via gluconic acid decomposition. This result is in agreement with the observation of Dutton and Evans (1996). They reported that D-glucose, D-xylose and D-galactose were better carbon sources than D-gluconate for oxalic acid production. The production of oxalic acid required a pH value close to neutrality and preferably at 6.0 pH. In all cultures studied the pH was kept at the level of 5.5 which

is the optimum for GOx production. Also, the accumulation of oxalic acid at high quantity is due to low decomposition of oxalic acid at this pH. The oxalate decarboxylase has been shown to be synthesized by *A. niger* only if the pH value of the culture medium was below 2.5 (Emiliani and Bekes, 1964).

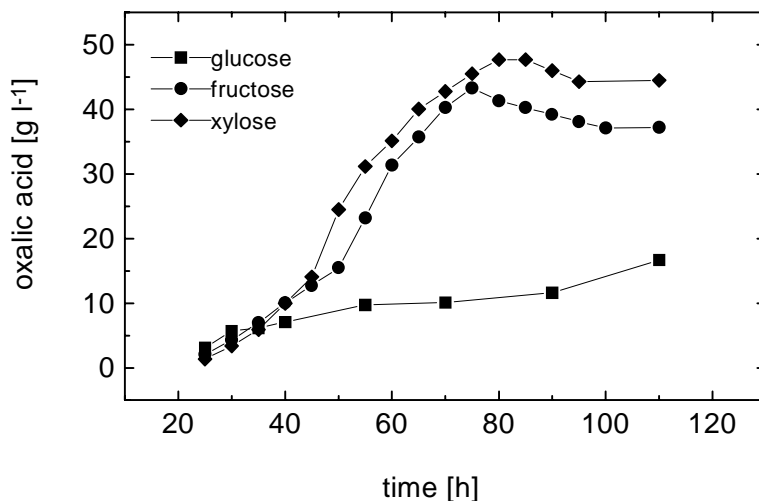


Figure 4.29: Oxalic acid production from different C-sources by *A. niger* during submerged cultivation on different C-sources.

In general, the production of oxalic acid in fungal cells is carried out from pyruvate through three different possibilities: by splitting of the oxaloacetate which does not enter the TCA cycle or formation of oxalate by splitting of oxaloacetate which arises from TCA cycle or formation of oxalate from glyoxylate via glyoxylate cycle (Dutton and Evans, 1996), **Fig. 4.30**.

In case of *A. niger*, the oxalate production is carried out mainly through the splitting of oxaloacetate in the cytoplasm. However, *A. niger* contains a cytoplasmic, constitutive pyruvate carboxylase, and it is therefore capable of forming oxaloacetate without the reactions of the TCA cycle (Kubicek *et al.*, 1988) .

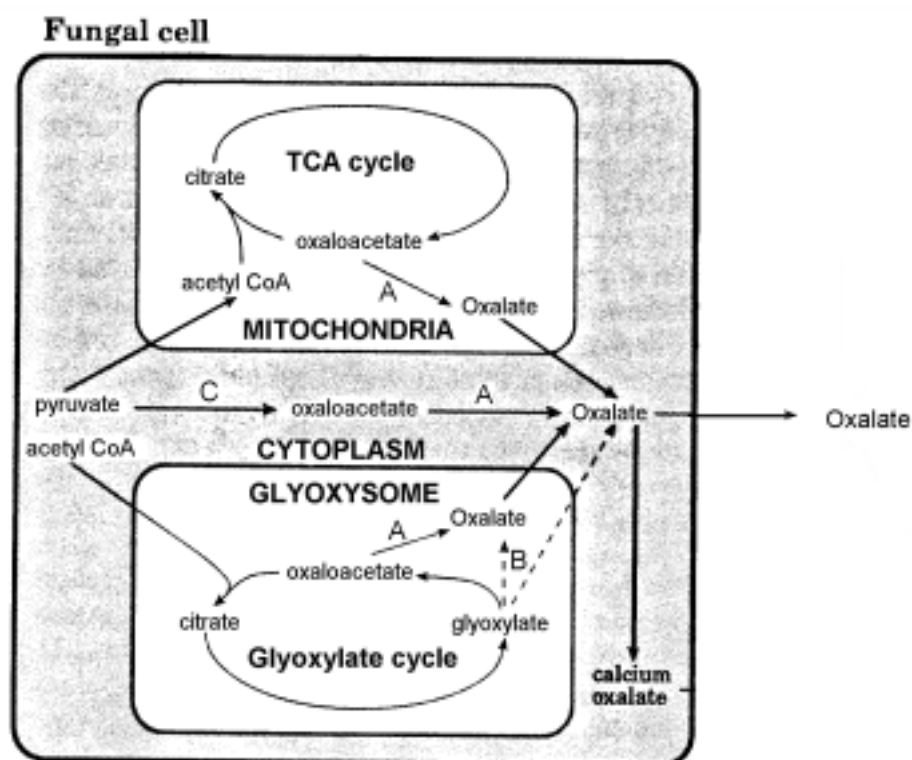


Figure 4.30: Different pathways involved in oxalate biosynthesis in fungal cells. A, oxaloacetase; B, glyoxylate dehydrogenase (glyoxylate oxidase); C, pyruvate carboxylase

Summary

From the previous results of the cultivations of the recombinant *A. niger* in different C-source cultures it can be concluded that:

- 1- The induction of GOx in a recombinant *A. niger* is possible using a non-glucose C-sources.
- 2- The morphology plays a significant role in both enzyme production and excretion.
- 3- For the comparison between the results of different cultures one should take in account the fungal growth morphology which may play a more significant role than medium composition or cultivation conditions.

4.6 Effect of different agitation speeds on cell growth and glucose oxidase production

4.6.1 Cell growth, substrate consumption and glucose oxidase production

The relations between cell growth, morphology and enzyme production and excretion as a function of different agitation speeds (200, 500 and 800 rpm) were studied. To prevent spore floatation and adhesion on the inner wall of the bioreactor, the agitation speed was 200 rpm during the first 5 h of cultivation and then adjusted for the desired speed and kept constant for the rest of cultivation time.

As shown in **Fig. [4.31]** the cell growth increased exponentially with the time in all cultures studied with more or less the same specific growth rate of about $0.08 - 0.09 \text{ h}^{-1}$ during the first 40 h of cultivation. In case of 800 rpm agitated culture no further increase in biomass was observed after 40 h and the cell concentration of about $16 \pm 1.0 \text{ g l}^{-1}$ was constant for the rest of the cultivation. On the other hand, in cultures with an agitation speed of 500 rpm the cell dry weight increased with time until it reached 22 g l^{-1} after 45 h and remained constant for the rest of the cultivation. In the 200 rpm culture the cell growth continued for longer time up to 60 h reaching about $29 \pm 1.5 \text{ g l}^{-1}$ and remained constant for the rest of cultivation time.

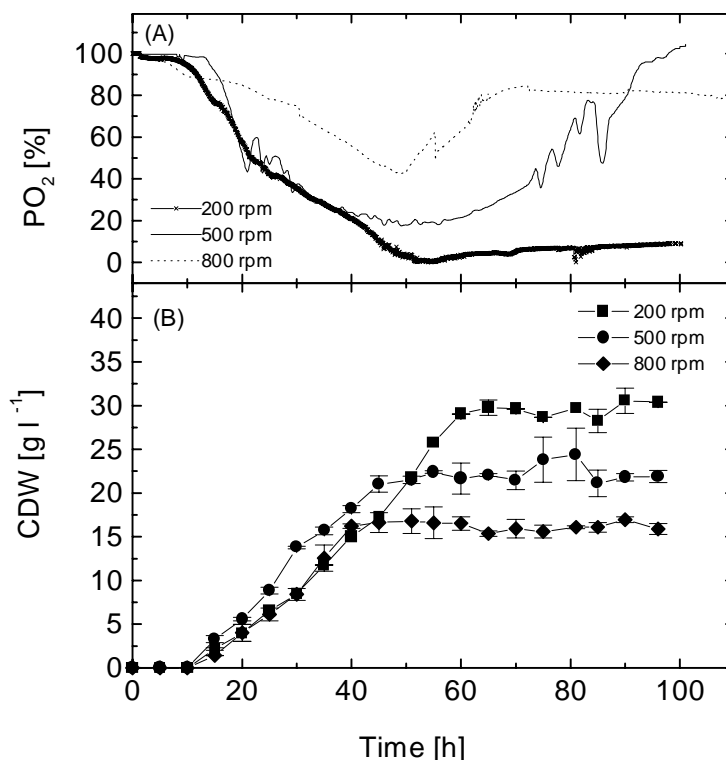


Figure 4.31: Effect of different agitation speeds on a recombinant *A. niger* growth in 5-L bioreactor (Data were taken from two different cultivations carried out under identical conditions).

Also a close relation between dissolved oxygen (pO_2) and growth was observed. The value of pO_2 was inversely proportional to cell growth. In the case of cell growth in the 200 rpm culture the pellet was less compact and composed of a long hairy hyphal thread in the outer layer of the pellet. After 70 h, growth was in a large non-compact pellet form connected with a mycelial network. This morphology increased the medium viscosity considerably which leads to a cell growth on the membrane of the oxygen electrode and the exact measurement of pO_2 value was not possible.

Although the agitation speed did not show a strong influence on growth rate during the first 40 h of cultivation, the agitation speed played a significant role in GOx production [Fig. 4.32].

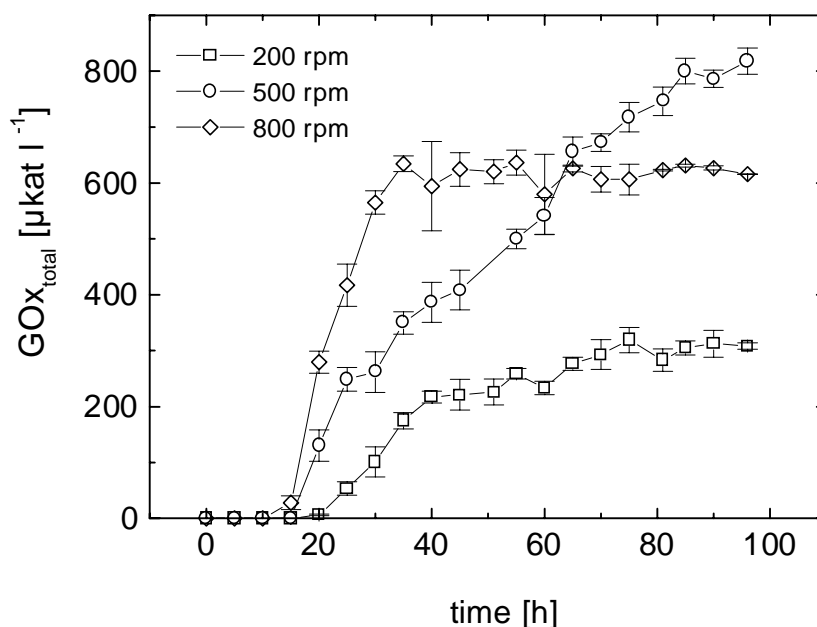


Figure 4.32: Effect of different agitation speeds on total GOx production by a recombinant *A. niger* in 5-L bioreactor (data were taken from two different cultivations carried out under identical conditions).

As shown in Fig 4.33, after a lag phase of about 10-15 h, GOx was produced with different rates depending on the agitation speed. As the agitation speed increased the amount of GOx increased concomitantly. The volumetric production rate of GOx [Q_{GOx}] shows a maximal value of about $50 \mu\text{kat h}^{-1}$ in case of 800 rpm agitation speed. Decreasing the agitation speed to 500 rpm the maximal production was decreased concomitantly to about $20 \mu\text{kat h}^{-1}$.

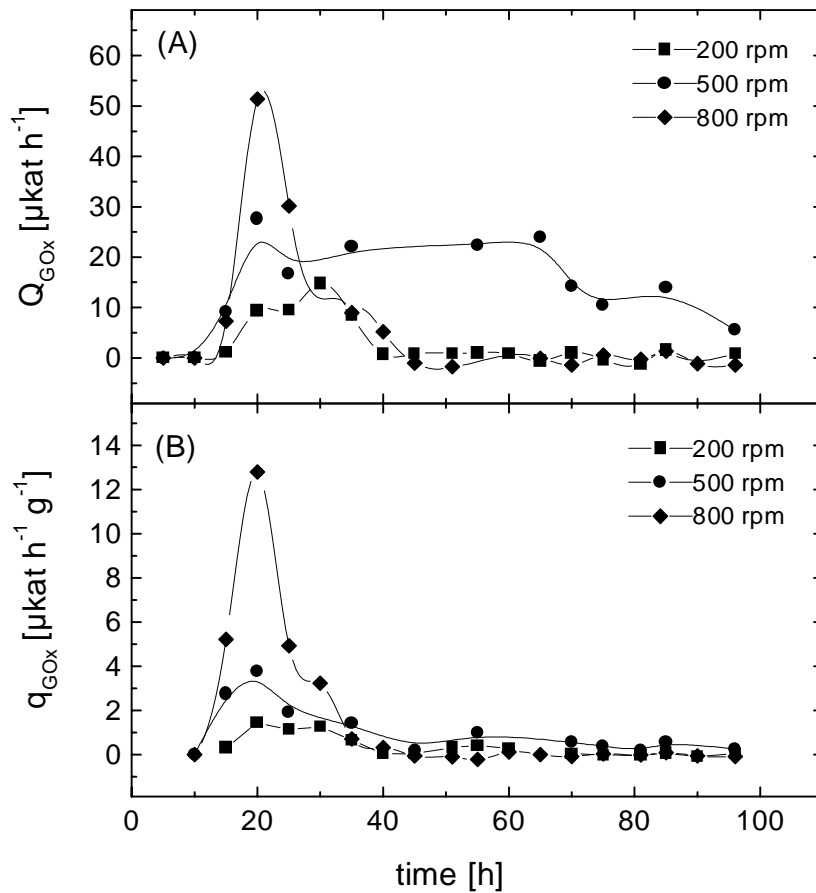


Figure 4.33: Glucose oxidase production rate by a recombinant *A. niger* as a function of different agitation speeds.

In the case of 200 rpm culture the maximal production rate of the enzyme of about $13 \mu\text{kat h}^{-1}$ was observed after 30 h. This rate decreased again with time and reached zero after 45 h. On the other hand, in cultures of 500 and 800 rpm the maximal enzyme production rate was obtained after 20 h of cultivation, 10 h prior 200 rpm, and in case of 500 rpm the production phase continued for longer time up to 55 h with a constant rate of about $21 \mu\text{kat h}^{-1}$. However, the longer production phase in 500 rpm culture was due to the formation of a new biomass (where the specific yield of enzyme production as $[\mu\text{kat h}^{-1} \text{g}^{-1}]$ decreased also after 20 h of cultivation). In case of 800 rpm the maximal GOx production rate was observed after 20 h reaching about $51 \mu\text{kat h}^{-1}$ and completely stopped after 45 h.

Since oxygen is one of the limiting reactant in the present process, a high level of dissolved oxygen will result in a higher oxygen uptake rate and thus a higher formation rate of GOx as in the case of high agitation speed culture. A higher oxygen uptake rate should lead to a more effective oxygen transport since oxygen was supplied continuously at a constant rate.

Because cell growth was completely stopped after 40 h in case of 800 rpm agitated culture and continued in other cultures, it was assumed that the production in other cultures will be higher as GOx is a growth-associated primary metabolite (Li and Chen, 1994). Contrary to this phenomenon, the GOx production under higher agitation speed was higher despite the lower biomass during the first 60 h of the cultivation.

The data presented so far show how the GOx production is influenced by the agitation speed but direct explanation of this phenomenon through the quantification of cell growth (CDW) was not possible (all cultures show almost the same CDW during the early 40 h). Likewise looking at the consumption of different substrates, i.e. carbon-, nitrogen- and phosphate- sources, no significant differences were observed in substrate consumption between these cultures during the first 40 h of cultivation [Fig. 4.34].

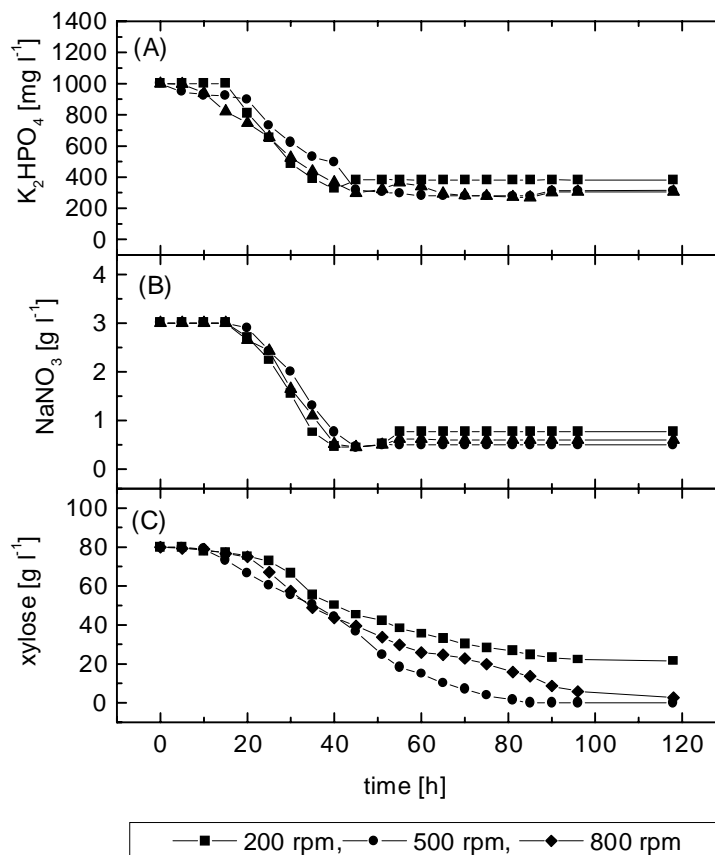


Figure 4.34: Effect of different agitation speeds on different substrate uptake during *A. niger* cultivation.

After 40 h the rate of xylose consumption in the 500 rpm culture increased with a higher rate compared to 800 and 200 rpm cultures. The lower consumption of xylose in 200 rpm observed was may be due to the growth morphology in the form of pellet (as only the outer zone was growing and

active). On the other hand, neither nitrate nor phosphate uptake had changed with different in agitation speeds.

The differences in enzyme production in these cultures were mainly due to two factors namely, the difference in oxygen transfer (which is dependent on the agitation speed), and the morphological feature of fungal growth. Thus quantification of cell growth using image analysis system was carried out in all cultures.

Because the first 40 h of cultivation were the most critical phase in this production process, the mechanism of growth during this period was extensively studied. The growth during this phase could be divided into three main steps:

1- Spore swelling and germination phase (0-14 h)

2- Hyphal tip elongation and branching phase (15-20 h)

(After 20 h of cultivation the growth shape was very complex and the micro-morphological feature could not be further followed).

3- Pellet formation and pellet growth phase (20 h - the end of cultivation)

4.6.2 Characterization of cell morphology under different agitation speeds

4.6.2.1 Spore swelling and germination phase (0-14h)

Inoculum in the concentration of 1×10^7 spores mL^{-1} was used in all cultures under study. At the beginning of the cultivation (t_0) not all spores were of the same diameter and normal distribution (which is good fitting with Gauss distribution) within the spore population was observed. In this cultivation system not only the mean volumes of spores might be adequate to represent the swelling process. Thus, the increase in spore diameter in the whole population was followed. Due to spore swelling in the early phase the mean diameter of spore increased gradually. Within the period of spore swelling spore diameter can be fitted to a normal distribution function [Fig. 4.35].

Before germination the spores first enlarge, swell gradually and increased in both diameter and biomass. During this phase of spherical growth, new wall layers are formed and laid down uniformly over the entire inner surface of the spores (Bosch *et al.*, 1995). In the experiment studied only spore swelling lead to an increase of about 100% in average spore volume during the first 5 h of cultivation. During this time the spore population was analyzed qualitatively, after staining with acridine orange (AO) and visualized under a fluorescence microscope. With this method a differentiation between spores undergoing swelling and latent one can be carried out.

The metabolic active spores give a red fluorescence (which indicates the presence of higher amounts of ssRNA and a minor quantity of DNA) whereas the latent or dormant spores give a green color

(which indicates the presence of high amounts of DNA and a minor quantity of ssRNA, Freudenberg *et al.*, 1996). At the time of inoculation (t_0) more than 80% of spores gave a green fluorescence after staining with AO. The amount of dormant spores decreased to about 30 % after only 2 hours of cultivation and disappeared completely after 3 hours. The relation between spore diameter and the color emission was also observed after this staining method. Generally, with few exceptions, no spores were observed giving green fluorescence beyond a diameter of 3.8 μm but not vice versa.

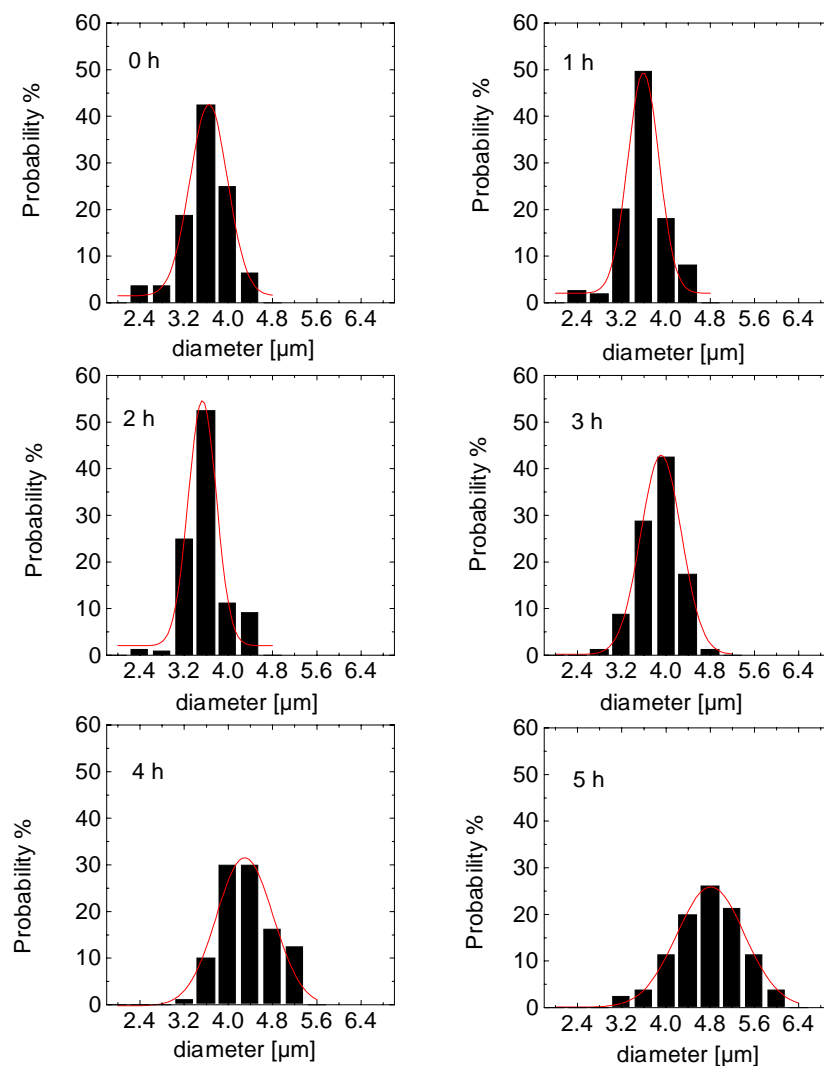


Figure 4.35: Swelling of conidiospores of a recombinant *A. niger* in 200 rpm xylose culture, as monitored at 1 h intervals by measuring the size distribution of the swelling spores by image analysis.

After 6h of cultivation, growth polarity was established and a hyphal element (germ tube) appeared from the enlarged spores. However, the germination of spores was found to be an asynchronous process depending on the viability and swelling rate of individual spore.

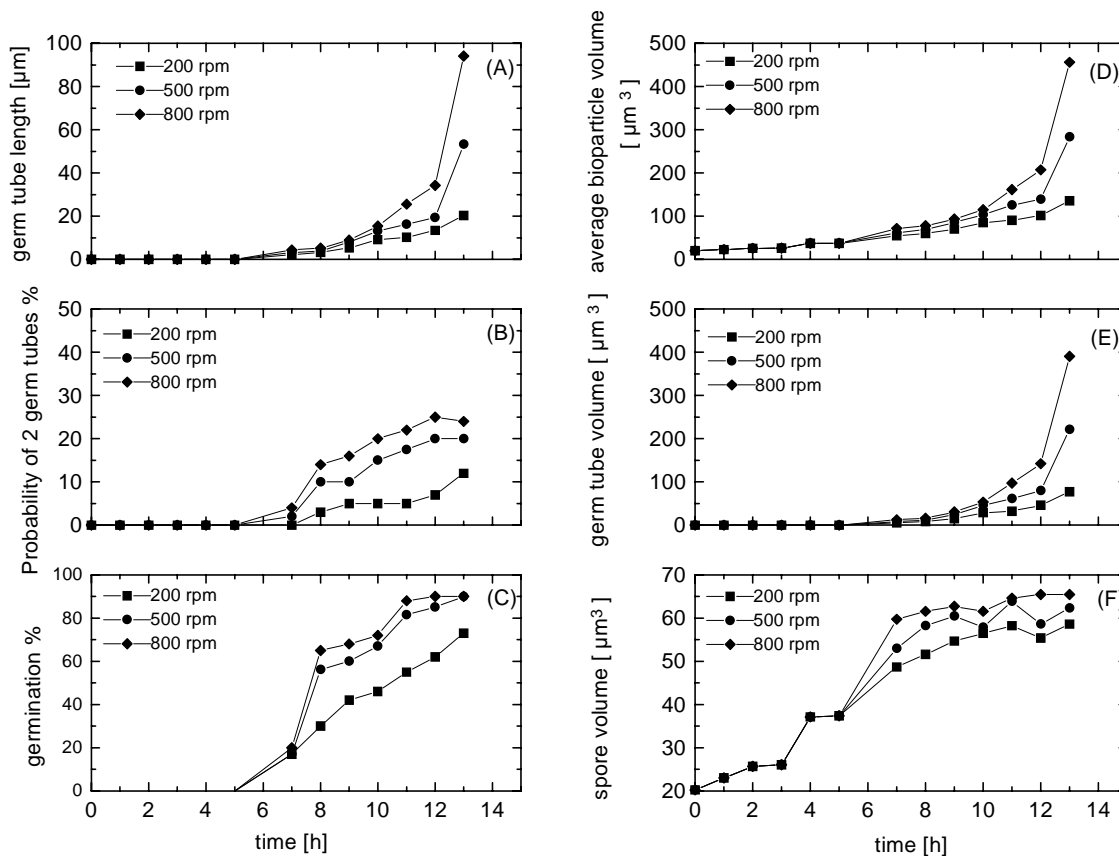


Figure 4.36: Effect of different agitation speeds on spore germination and germ tube elongation during cultivation of a recombinant *A. niger* in submerged culture (A-C). The increase in bioparticle volume with time (D-F).

Figs. 4.36B and C show the percentage of germinated spores during cultivation at three different agitation speeds. The agitation speed showed a significant effect on spore swelling and germination as well. The proportion of the germinated spores (either one or more germ tube per swollen spore) and the time of germ tube formation were influenced by the agitation speed. It showed that at 12 h, 90% of spores had germinated in the 800 rpm culture compared to only 79% and 62% for 500 rpm and 200 rpm cultures, respectively.

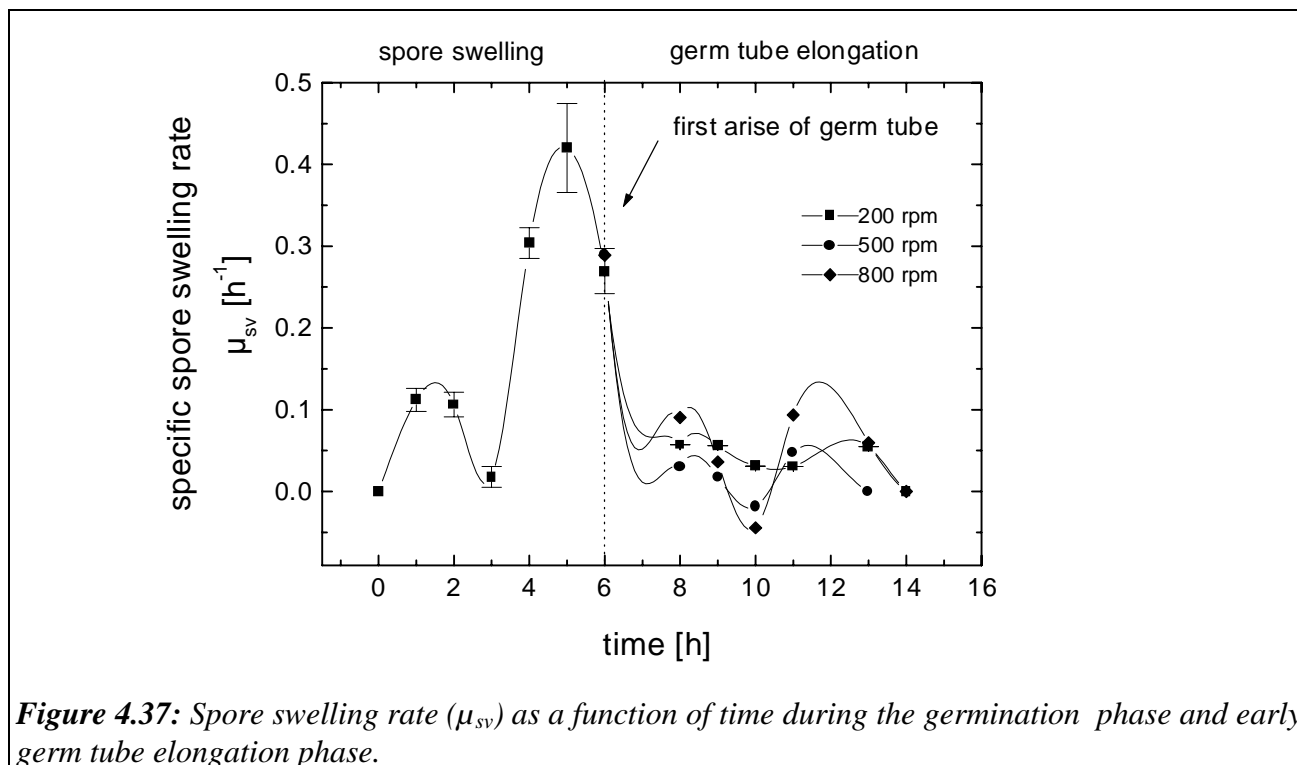


Figure 4.37: Spore swelling rate (μ_{sv}) as a function of time during the germination phase and early germ tube elongation phase.

As clearly observable in **Fig. 4.36C** spores germinated after a lag phase of about 5 hours. During this time an increase in spore diameter due to swelling was carried out as previously described.

The mean volumes of non-germinated and germinated spores are presented in **Fig. 4.36F**. Spherical growth of the spores progressed with further incubation. The intensity of agitation had a significant effect on spore swelling (growth) and their eventual size, with a higher growth in the highly agitated culture. To get a better understanding of the swelling process during this phase, the calculation of specific spore swelling rate (μ_{sv}) was done. As shown in **Fig. 4.37** an increase in the specific swelling rate was observed during the first 3 hours (after this time no more green colored spores were observed), a dormant time, this increase in swelling rate is mainly due to physical swelling and water uptake. After that another peak of the specific swelling rate was observed driven by active swelling (due to biological activities resulted in an increase in the cytoplasmic pressure inside the spore) and after 6 hours an obvious decline in this peak was observed (the first rise of germ tube, which decreases the internal pressure inside the spores).

The germination characteristics of fungal spores have been discussed by Wastie and Janardhanan (1970). They studied the new cell layer formations and cytological changes of the spores during

germination. They reported that the dormant spore shifts from low to high metabolic activity during the germination process. This process starts automatically if spores are placed in suitable environmental conditions. Also, dormancy can be interrupted by an activation process such as heat shock or by chemical treatment. The swelling and germ tube emergence in the germination process constitute a major part of the lag phase in cultivations inoculated with spores. The factors affecting spore germination in fungi were discussed by many authors as a function of different factors such as: age of spores used as inoculum (Paul *et al.*, 1993), medium composition such as C-source, N-source and other medium ingredients (Campbell, 1971; Tripp and Paznokas, 1981; Paul *et al.*, 1993) and elevated temperature (Anderson and Smith, 1971).

In our study, the relation between the agitation speed and the kinetics of the spores germination was observed. The increase in the spore swelling rate with the increase in agitation speed may be attributed to the rate of the dissolution of the outer hydrophobic layer which prevents spores from dehydration. This outer (rodlet) layer of spore consisting of hydrophobic protein and melanin works as a barrier against mass transfer from the medium to the inside of the spore resulting in a reduction in swelling and germination (Claverie-Martin *et al.*, 1986; Wessels, 1997). Moreover, this layer plays a significant role in aggregation between spores.

However, spore activation is dependent on the penetration of both oxygen and nutrient from the surrounding medium to the inside of the spore because the spores of many fungi undergo a rapid increase of the oxidative respiration during spore germination (Campbell, 1971).

Thus, before germination of spores in liquid medium, dissolution of this soluble protein should be carried out and the rate of swelling will be dependent on the rate of solubilization of this layer which results in a decrease of hydrophobic properties of spores. The dissolution of this layer may be achieved either by shaking or homogenization of spores (Claverie-Martin *et al.*, 1986; Muñoz *et al.*, 1995). The different stages of spore swelling and germination are represented in **Fig. 4.38** as observed under fluorescence microscope .

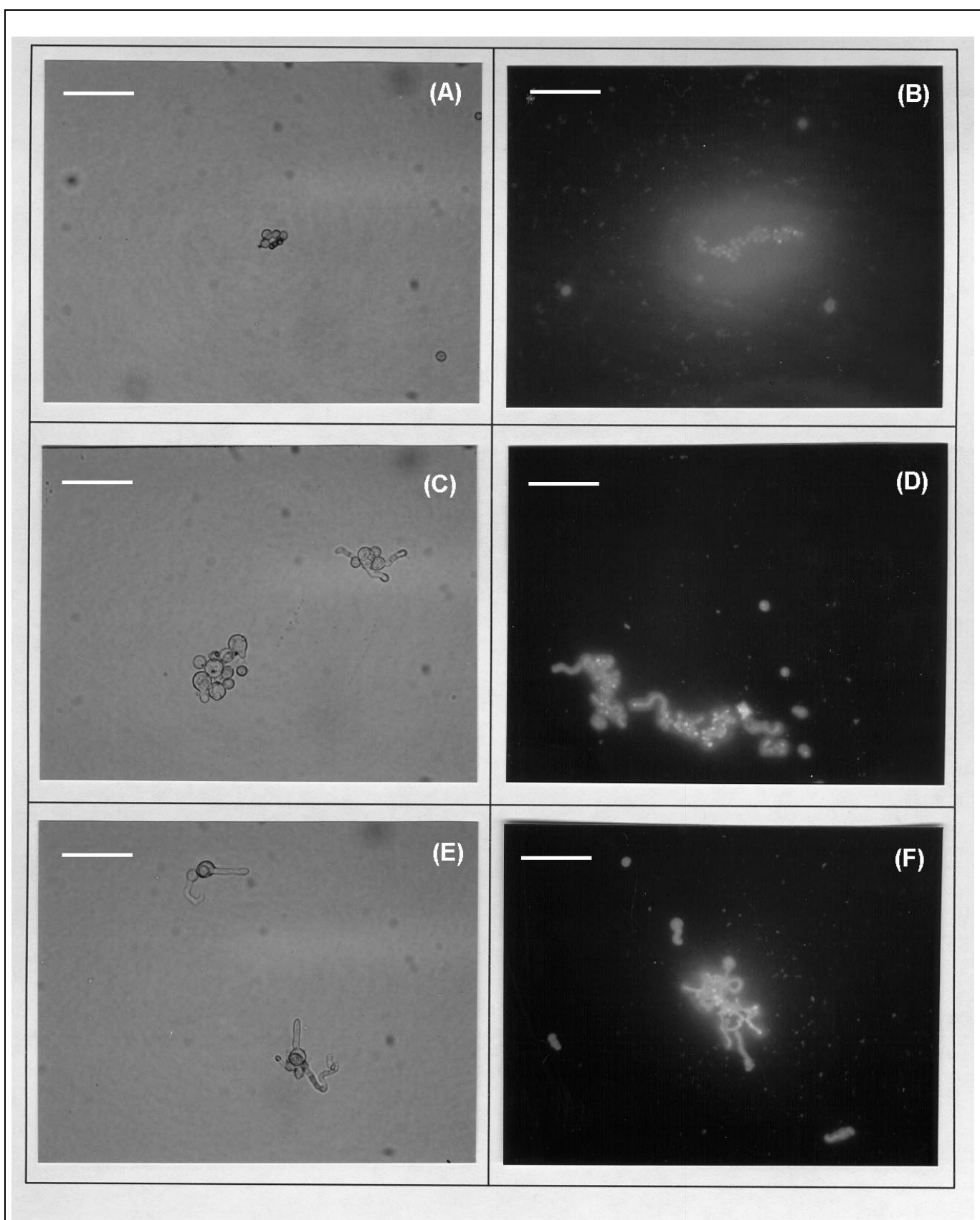


Figure 4.38: Spore swelling and germination during the early cultivation time. (A and B) show non-germinated spores after 5h of inoculation. (C and D) show germinated spores and the development of germ tube after 7 h. (E and F) show elongated germ tube after 9 h. (bar = 40 μ m).

4.6.2.2. Hyphal tip elongation and branching phase (15-20 h)

In the presence of excess nutrients, suitable environmental conditions and the absence of inhibitors, biomass of unicellular microorganisms increase exponentially. This is not possible for a germ tube hyphae extending at a constant rate without an increase in diameter, as it does not normally occur. In addition, the cytoplasm which is synthesized in distal regions, but which is unable to reach the hyphal apex, must be accommodated in the same way. Both of these problems are solved by the formation of branches (Prosser and Tough, 1991). The first branch is usually formed before the germ tube hyphae achieves a constant extension rate. Continued branch production then results in an exponential increase in total mycelial length and number of branches at the same specific rate.

During this phase the measurement of newly formed vegetative growth was followed through the hyphal cell elongation. Branching started after 14 h when the hyphal elements have reached an average length of about 150-200 μm . During the time of branching the hyphal extension rate decreased [Fig. 4.39].

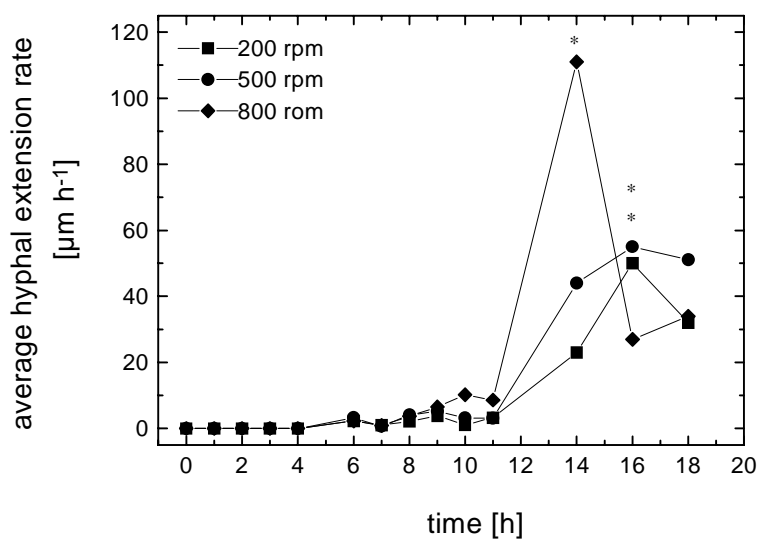


Figure 4.39: Hyphal extension rate as a function of different agitation speeds.
(*) indicates the first arise of branching.

However, branching may result from a high biomass formation and growth rate in the distal part of the mycelium, increasing the internal pressure in this region and resulting in branching.

Bioparticle volume increase rate in micro-morphological level

Fig. 4.40 shows three main peaks in the specific bioparticle increase rate $[\mu_{bp}]$. The first small peak was after 1-2 h due to the physical swelling of spores. Swelling resulted from spore growth gave the second peak during the time between 4-6 h and decreased at the time of germ tube formation (6 h). From 7 to 10 h the increase in $[\mu_{bp}]$ was constant and it increased again prior to the time of branching which was at 14 h in case of 500 and 800 rpm cultures and at 16 h in case of 200 rpm culture.

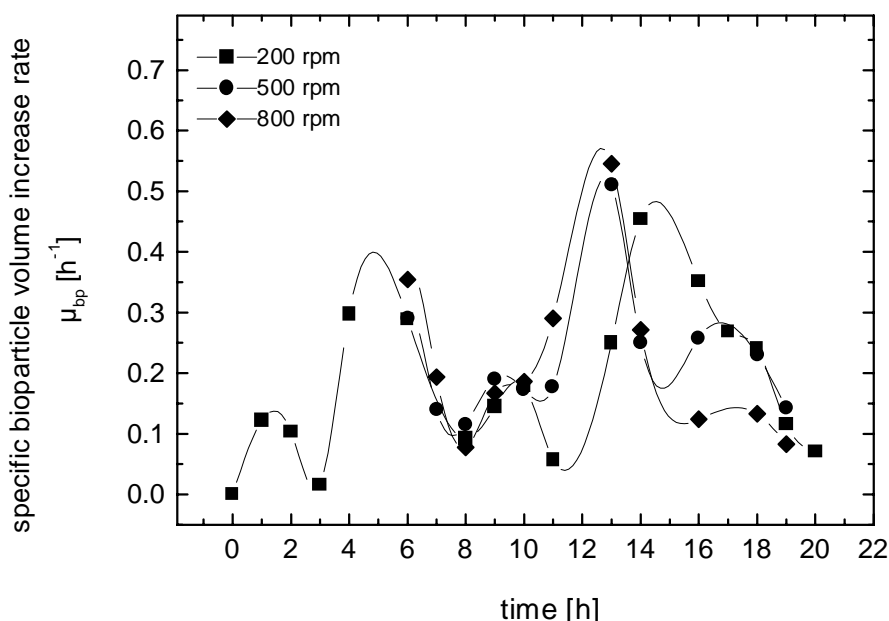


Figure 4.40: Specific bioparticle volume increase rate (μ_{bp}) with time under different agitation speeds.

Note: Bioparticle volume is the total volume of spore, germ tube and branch(es).

Generally, we can conclude that the observed peaks of $[\mu_{bp}]$ value were at the time of maximal pressure inside the bioparticle before finding a solution to decrease it (germ tube formation or branching). It is also worth to note that agglomeration between the grown bioparticles was observed during this phase which may interfere with the obtained results.

4.6.2.3 Pellet formation and pellet growth phase (20 h - the end of cultivation)

After about 20 h of the cultivation, agglomeration between the hyphal elements was very high and the growth form was characterized as pellets. In case of 200 and 500 rpm cultures a distinct pellet morphology was observed with small fraction of free mycelium that decreased with the time. However, the change in aggregate/pellet diameter was followed in these cultures. **Fig. 4.41**

demonstrates a negative relation between the agitation intensity and aggregate/pellet diameter. On the other hand, the number of aggregate/pellet increased with the increase of the agitation speed. The number of pellet was of 45 ± 5 and 250 ± 40 pellets ml^{-1} in 200 and 500 rpm agitated cultures, respectively.

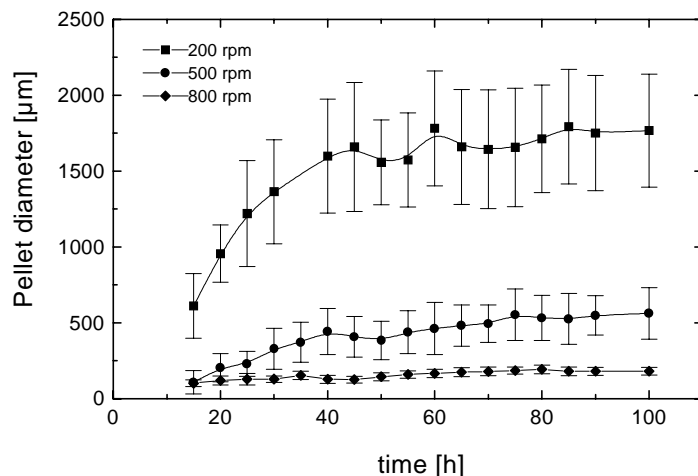


Figure 4.41: Change in aggregate/pellet diameter during cultivation of *A. niger* in 5-L bioreactor under different agitation speeds.

During the time period of 15 to 40 h the average pellet diameter increased with a constant rate of 39.49 and 13.39 $\mu\text{m h}^{-1}$ for 200 and 500 rpm agitated cultures, respectively.

However, the determination of the average pellet diameter was not sufficient to quantify the growth morphology and the active fraction of the biomass. Therefore, the pellet size distribution was determined in each samples from 100-150 bioparticles. The change of pellet diameter in each populations during cell cultivation is represented in **Figs. 4.42** and **4.43** (see also **Tabs. 9.2.2** and **9.2.3, Appendix**). The growth under agitation speed of 800 rpm was mainly in mycelial network with a higher number of aggregates with small diameter. After about 40 h, the fraction of free mycelial cells decreased through the fragmentation of cells in this culture. As shown previously, the increase of agitation speed inhibited the aggregation between the germinated bioparticles which resulted in a significant decrease in pellet diameter. Therefore, the observed differences in Gox production could be attributed to the shear force in the bioreactor and the resulting morphological differences during the cultivation.

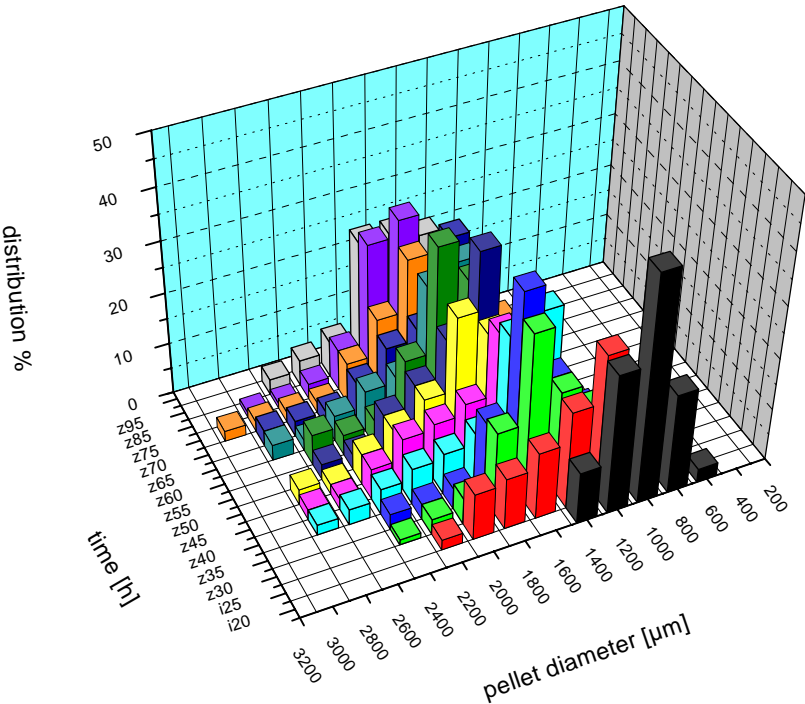


Figure 4.42 : Distribution of pellet diameter during cultivation in 5-L bioreactor at 200 rpm.

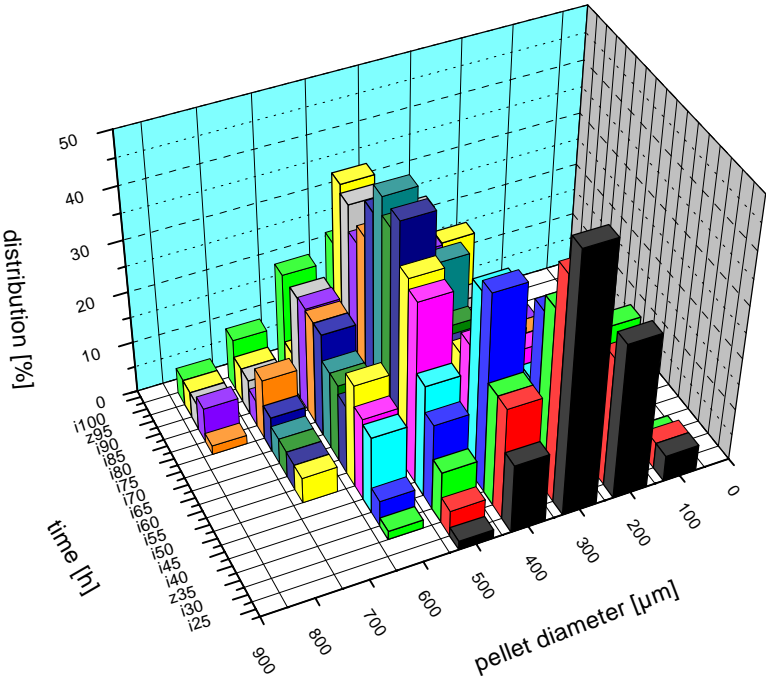


Figure 4.43: Distribution of pellet diameter during cultivation in 5-L bioreactor at 500 rpm.

4.6.3 The relation between growth morphology and the yield of glucose oxidase production

To get a better understanding of the relation between the hydrodynamic stress, morphology and enzyme production, the yield of enzyme produced per biomass was calculated. Generally, the yield of enzyme production ($Y_{P/X}$) increased proportionally with the agitation speed [Fig. 4.44].

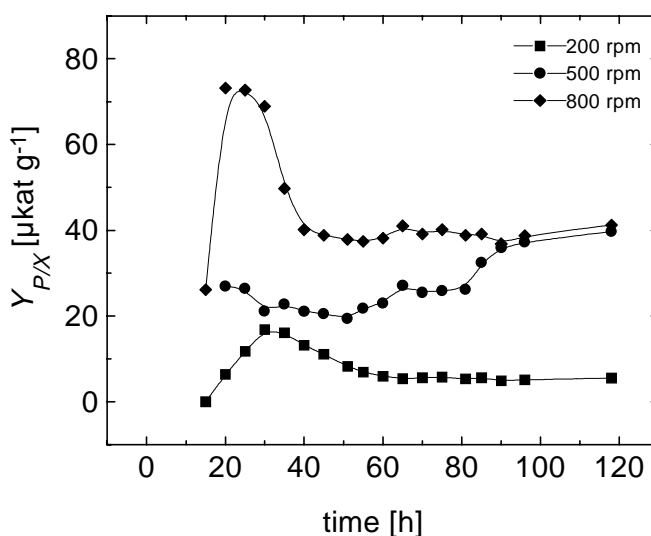


Figure 4.44: Yield coefficient of enzyme production during cultivation of a recombinant *A. niger* under different agitation speeds.

In case of 800 rpm agitation culture, the growth was mainly in micropellet-filamentous form and all cells were grown and were active (as observed by fluorescence microscope [Fig. 4.45]). On the other hand in 200 rpm agitated culture, that grew in large pellet form, not all cells were active and the growth and enzyme production were only restricted to the outer layer of the pellet. This outer layer was found to be about 100 μm in thickness. In 500 rpm agitated culture growth was in the form of small pellets with mycelial network. Also, a small fraction of non-growing cells was observed but the quantification of this fraction was not determined due to the high complexity of growth.

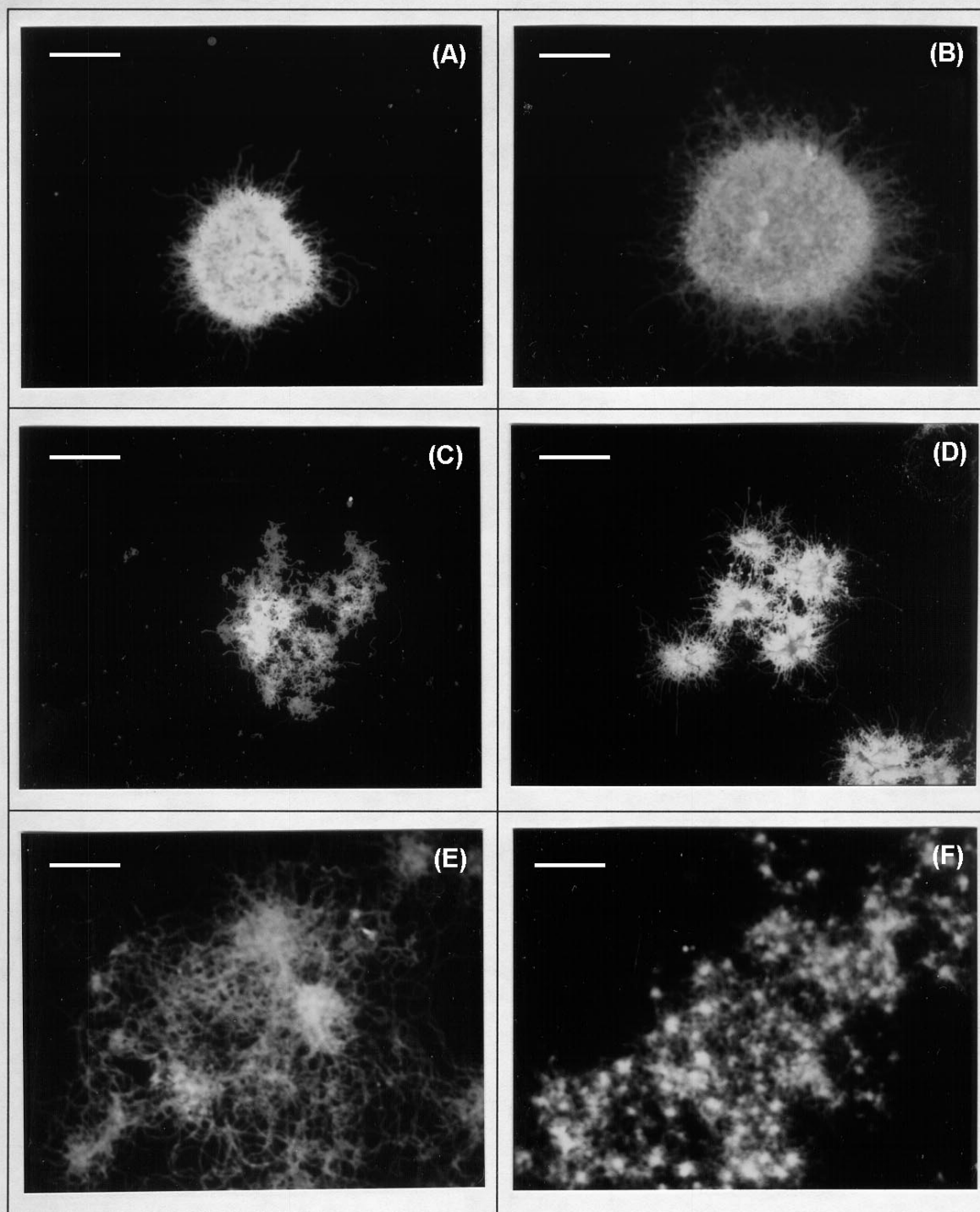
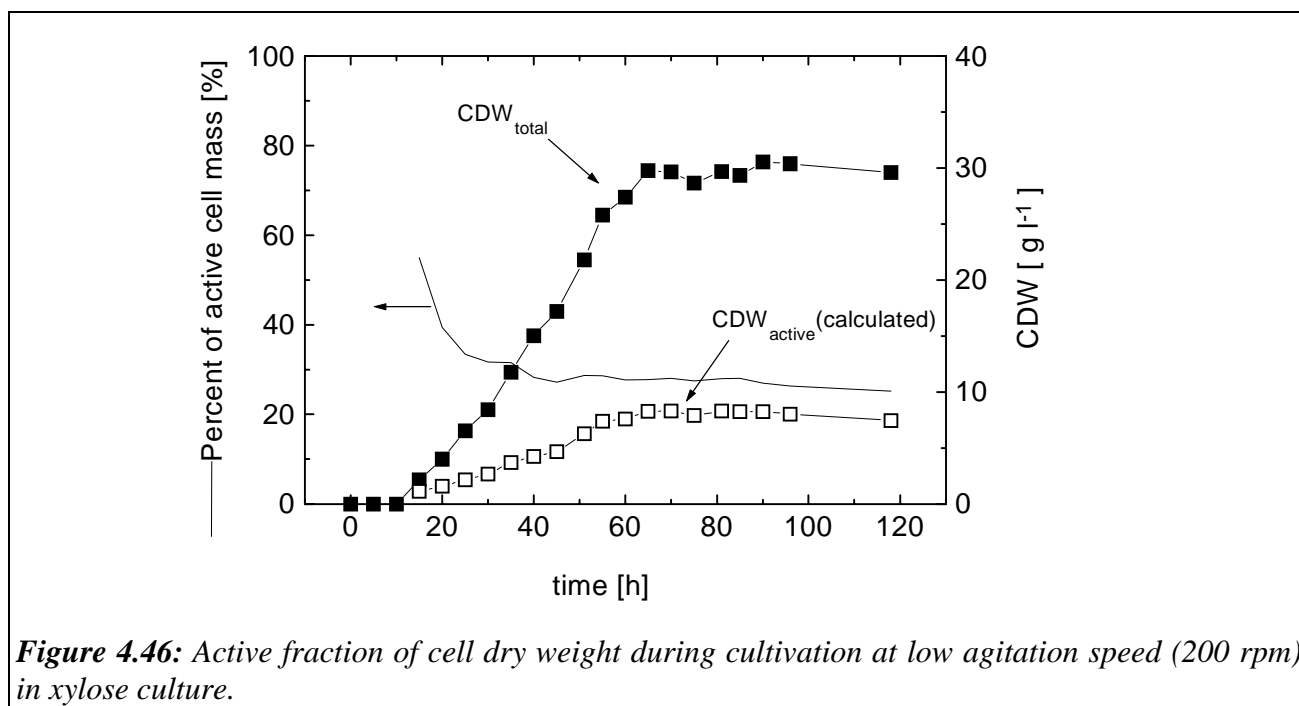


Figure 4.45: The change in growth morphology with the time under different agitation speeds. (A, C and E; after 19 h) and (B, D and F, after 25 h) for growth under agitation speeds of 200, 500 and 800 rpm, respectively. For A, B, C, D and F (bar = 300 μm) and for E, (bar = 75 μm).

The active fraction of biomass in case of 200 rpm was calculated according to the **Eqs. 3.10-3.19**, (materials and methods) taking the pellet distribution in consideration [**Fig. 4.42**] and [**Tab. 9.2.2., Appendix**]. However, it is of importance to determine the pellet distribution in culture which defines the amount of mycelium in contact with the growth medium and hence the amount of growing mycelia.

As shown in **Fig.4.46** only 30% of biomass was active after 25 h of cultivation. The active fraction of biomass was more or less constant during the rest of cultivation time.

For the growth in 500 rpm culture, the quantification of the active biomass was difficult because growth was in a mixture of pellet and filamentous form.



After calculation of the active biomass yield in case of the 200 rpm culture [**Fig. 4.46**], the specific yield of enzyme was equal to the value of 800 rpm culture. i.e. the production of enzyme was mainly associated with the outer growing layer of pellet [**Fig. 4.47**].

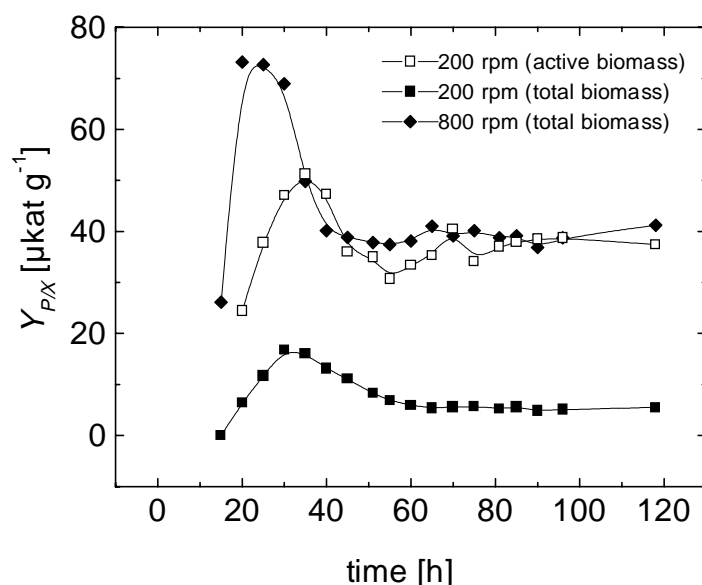


Figure 4.47: Yield coefficient of enzyme production during cultivation of a recombinant *A. niger* in 200 and 800 rpm agitated cultures.

4.6.4 Glucose oxidase excretion under different agitation speeds.

The GOx excretion under different agitation speeds is shown in **Fig. 4.48**. During the high production phase, the first 40 h, the accumulation of extracellular enzyme increased with the time. The rate of enzyme excretion also increased as a function of increasing the agitation speed and were 2.37, 5.14 and 7.08 $\mu\text{kat l}^{-1} \text{h}^{-1}$ in 200, 500 and 800 rpm cultures, respectively. The differences in enzyme excretion rate were due to the morphological differences during this phase either in micro-morphological form (hyphal cell elongation and branching) or in macro-morphological form (aggregate shape and diameter). The growth under high agitation was characterized by a higher hyphal extension and branching. Therefore, it showed a higher number of apical tips which lead to an increase in the enzyme excretion. Also the increase of the agitation speed inhibit the aggregation of both spores and hyphal cells resulting in a micropellet growth in hyphal network. This form of growth is characterized by a higher surface area and a better contact between the cells and the cultivation medium.

The positive relation between internal metabolite excretion and agitation speed in filamentous fungi was also observed by other authors. Tanaka *et al.* (1974) studied the leakage of different intracellular substances during agitation of mycelial suspension of *Mucor javanicus*.

They found that the amount of metabolites leaked (RNA related low molecular weight nucleotides and protein related substances) is directly proportional to the agitation intensity. This phenomenon was not caused by destruction of mycelia. Also, Ujcová *et al.* (1979) reported that the release of nucleotides from *A. niger* cells increased with the agitation speed. Morimura *et al.* (1992) observed that the release of nucleotides in *Aspergillus* culture is not only dependent on the agitation speed but also on growth morphology. The leakage of intracellular nucleotides, during cultivation at the same shear stress, was higher in filamentous form compared to pellet form.

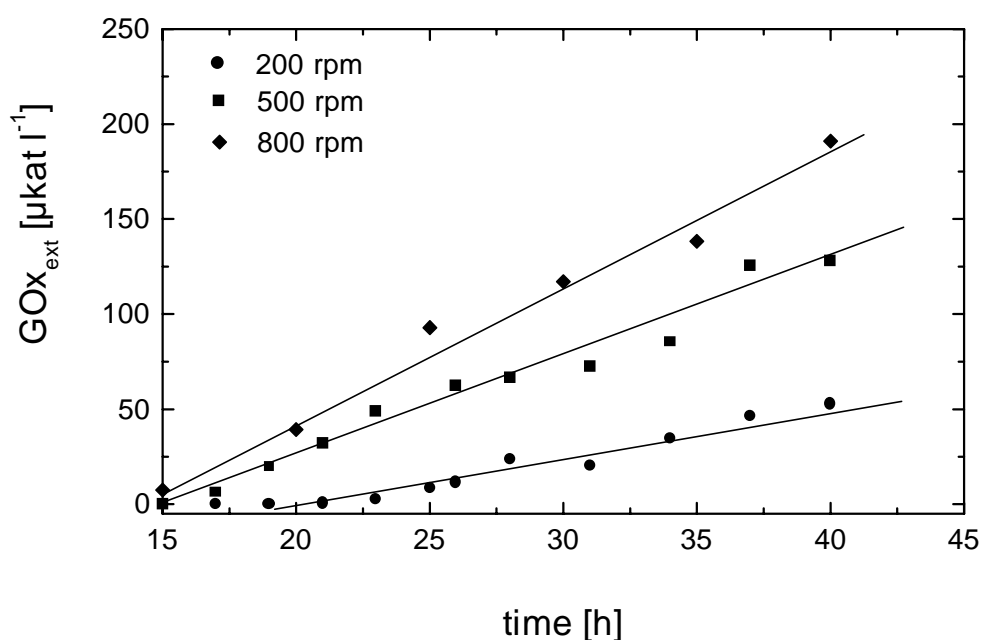


Figure 4.48: Enzyme excretion during cell cultivation under different agitation speeds.

It is worthy to note that the intensity of red color in the apical tip region after staining with AO decreased with the time [Fig. 4.49]. This may give an indirect indication of the enzyme excretion in this region, because the red color after AO staining is a sign of the presence of high ssRNA content.

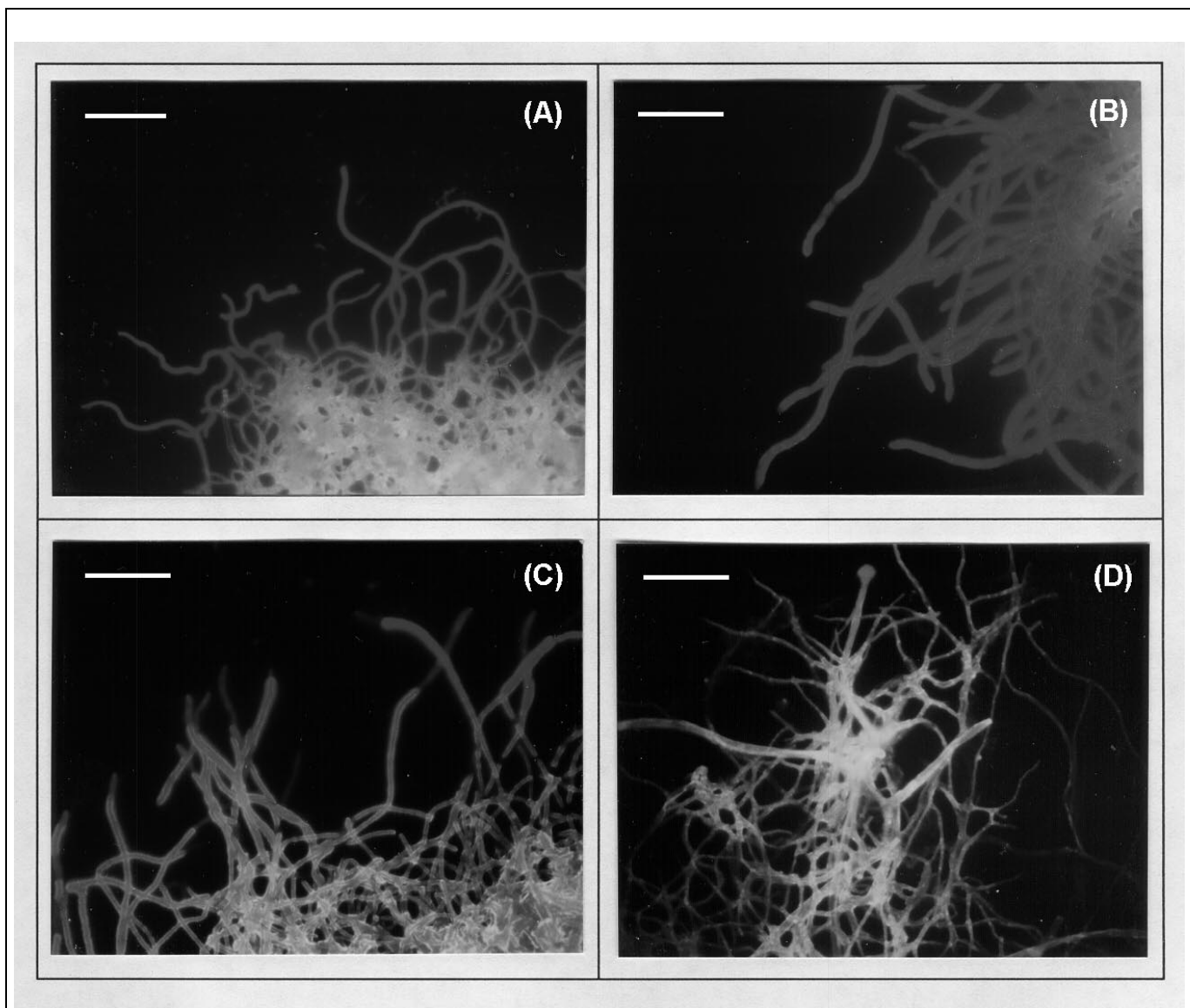


Figure 4.49: Apical tip of fungal hyphae during cell cultivation in submerged culture as observed under fluorescence microscope after staining with AO. (A) apical tip in 15 h old culture (bar = 75 μm); (B) apical tip after 19 h (bar = 47.5 μm); (C) apical tip after 23 h (bar = 37.5 μm); (D) apical tip in 50 h old culture (bar = 75 μm).

4.6.5 Xylitol and oxalic acid production

In all cultures studied xylitol was formed as an intermediate product. The amount of produced xylitol increased proportionally to the agitation speed [Fig. 4.50]. After about 25 h, xylitol was first detected in the cultivation medium and reached to a maximum of 5.2 and 6.6 g l⁻¹ after 45 h in 500 rpm and 800 rpm agitated cultures, respectively. After this time, the xylitol concentration decreased gradually and completely disappeared after 70 h. In case of 200 rpm culture, xylitol was first detected after 35 h reaching a maximum of 2.1 g l⁻¹ after 40 h and disappeared completely after 55 h.

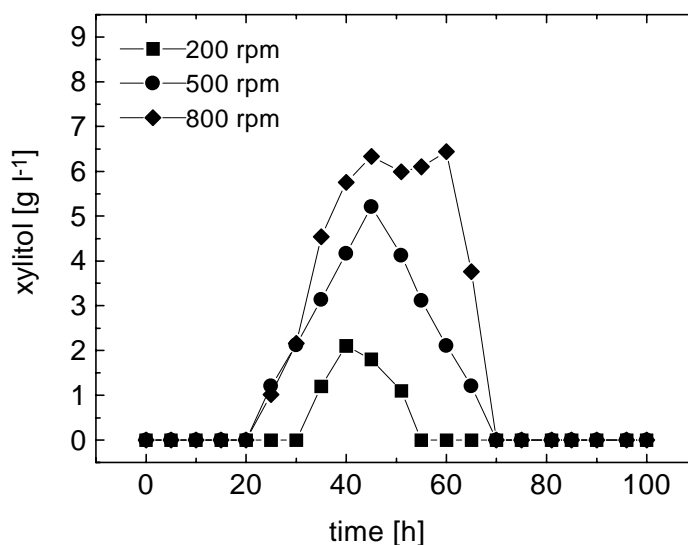
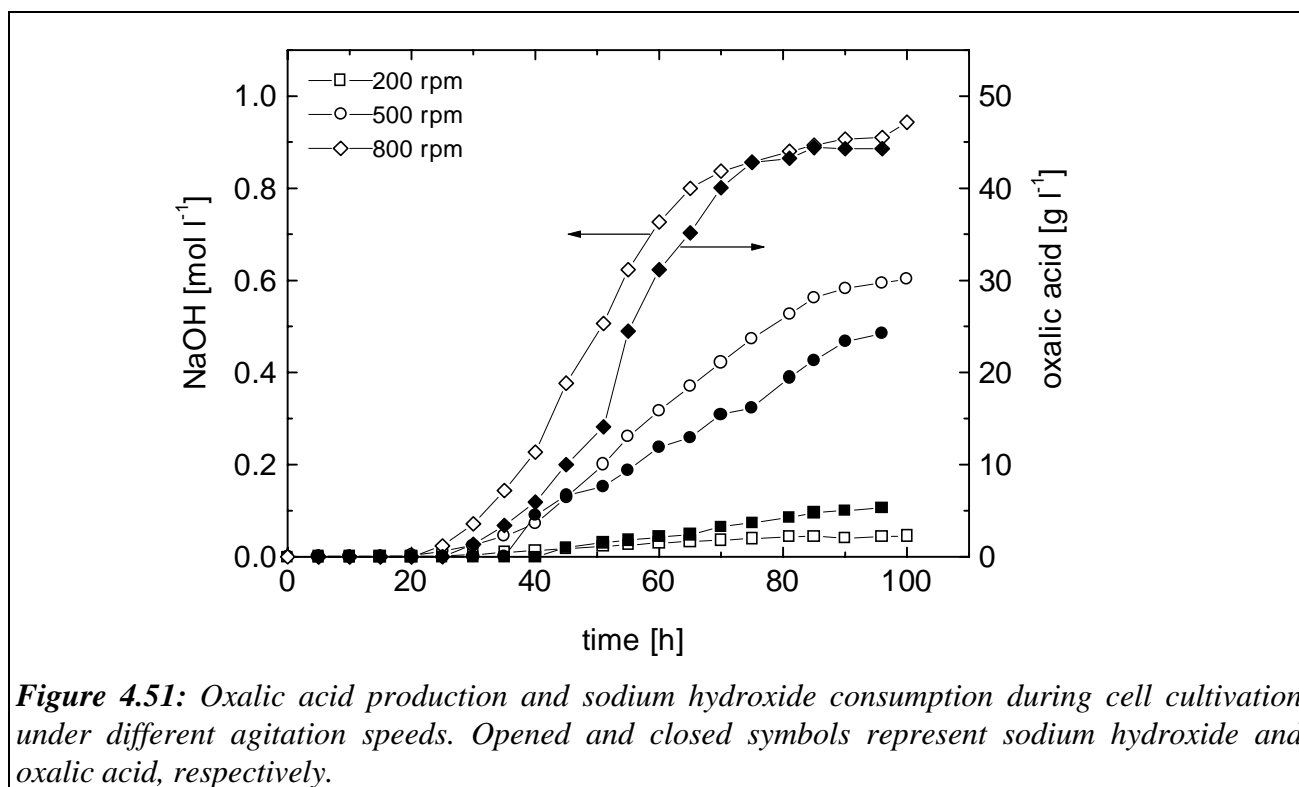


Figure 4.50: Xylitol production during *A. niger* cultivation under different agitation speeds in xylose cultures.

During all cultivations studied sodium hydroxide was required to neutralize the pH of medium and keep it at the level of pH 5.5 (the optimal pH for GOx production). The amount of sodium hydroxide required was directly proportional to the amount of oxalic acid produced. Fig. 4.51 shows the production of oxalic acid and sodium hydroxide consumption in cultures under different agitation speeds.



As shown the accumulation of oxalic acid in *A. niger* culture was highly dependent on the agitation speed. The production reached about 30 g l⁻¹ and 45 g l⁻¹ in culture agitated with 500 and 800 rpm, respectively. On the other hand, at 200 rpm agitation speed only about 5 g l⁻¹ of oxalic acid was produced.

4.6.6 Carbon balance

The carbon balance was determined in all cultures according to the simplified equation [Eq. 4.3] as described previously. **Fig. 4.52 A, B and C** show the carbon balances of the cultivations under different agitation speeds of 200, 500 and 800 rpm, respectively. The carbon recovery was close to 100% in most cases. Deviation in the value of C-recovery may occur that may due to be a small error in biomass determination which is within the range of 5 – 10%. As shown, not only the production of GOx was influenced by the agitation speed but also the mode of utilization of the C-source, xylose, for biomass, xylitol, oxalic acid and carbon dioxide formation as well. In spite of the higher biomass formation in 200 rpm agitated culture, the amount of carbon dioxide produced was less compared to other cultures. This result supports also our conclusion of the lower metabolic activity in case of growth in low agitated speed culture due to the large pellet formation.

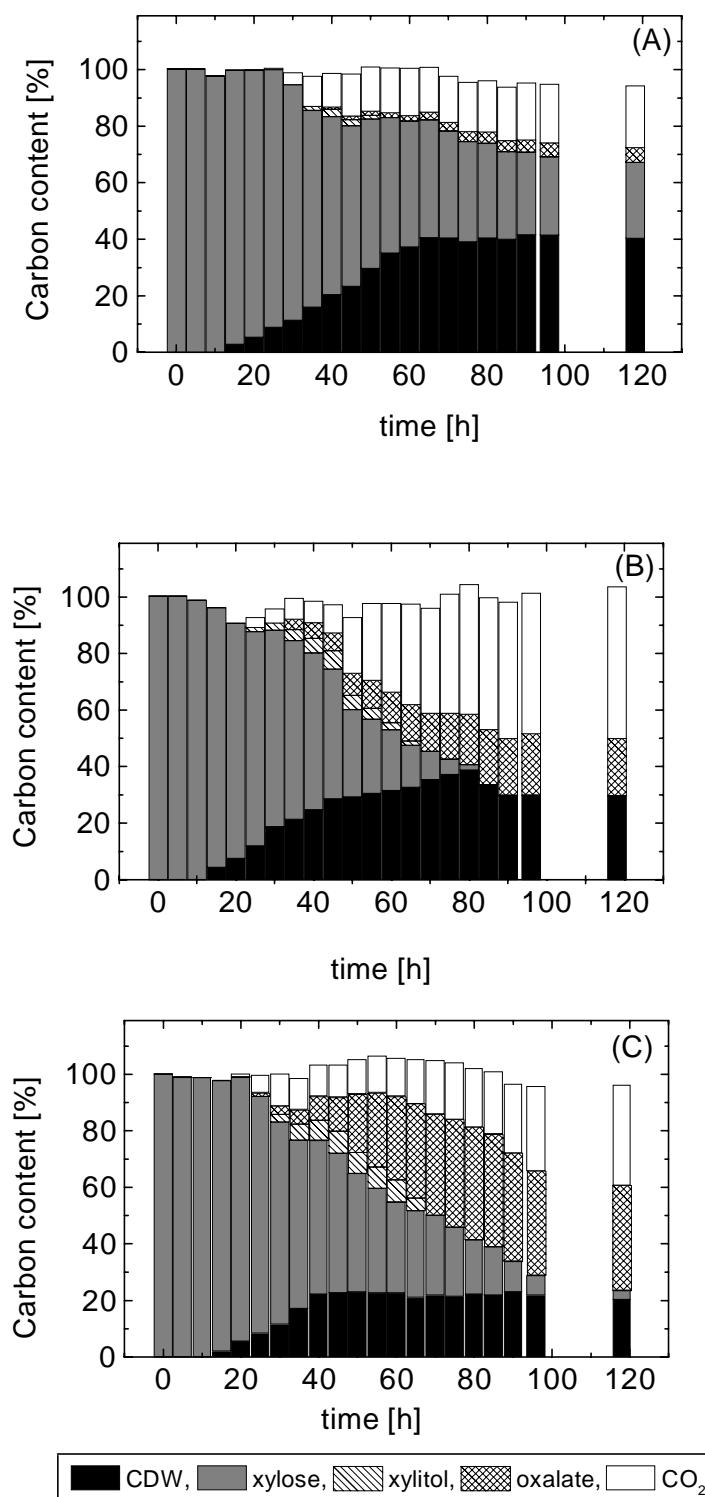


Figure 4.52: Carbon balance during cultivation under different agitation speeds. A, B and C represent cultivations under agitation speeds of 200, 500 and 800 rpm, respectively.

As shown previously a tight relation was observed between the agitation speed and GOx production. Roukas (1991) found that the activity of aconitase and isocitrate dehydrogenase increased with the increase of agitation, while the activity of citrate synthase decreased with the increase in agitation speed. Therefore, we can conclude that the effect of the agitation speed on the enzyme production is dependent on the type of enzyme under study.

As demonstrated in this study, the cultivation at a moderate agitation speed of 500 rpm was better for volumetric production of GOx. This result is in agreement with Zetelaki and Vas (1986) who found that the GOx production in the wild type *A. niger* increased considerably with the increase in agitation speed from 470 to 700 rpm. When the speed of agitation was increased to 940 rpm, both of growth and total GOx were somewhat lower than in culture agitated at 700 rpm. Also, Petruccioli *et al.* (1995) studied the influence of stirring speed ranged from 300 to 900 rpm on GOx production by *Penicillium variable*. They observed a maximal enzyme production in 400 rpm agitated culture. On the other hand, it has been shown in our study that the cultivation in 800 rpm culture resulted in a higher production and excretion rate of the enzyme during the first 40 h. The termination of enzyme production after 40 h in the case of 800 rpm might have resulted from cell damage by the high shear stress. When the biomass eventually reached a maximum value and the culture entered the stationary phase, a relatively constant biomass was maintained through a balance between growth and autolysis. This kind of balanced growth in the case of hyphal cell growth under high shear stress was also reported by Prosser and Tough (1991).

To overcome this problem cultivations were continued with two main strategies:

- 1-Cultivations with the addition of a biopolymer (xylan) were done to minimize the destructive effect of the hydrodynamic stress on the cells during cultivation under high agitation speeds.
- 2-Cultivation in fed-batch culture to increase the cell mass through exponential feeding of the consumed substrates.

4.7 Production of glucose oxidase in mixed substrate (xylose/xylan) cultures

The first step in this experiments was the cultivation of a recombinant *A. niger* in shake flask culture with different ratios of xylose/xylan. This experiment was done to investigate the effect of this biopolymer on cell growth, enzyme production in shake flask and the possibly to substitute xylose with cheaper pentose polymer.

4.7.1 Shake flask experiments

In this experiment cultivations were done in baffled shake flasks using a basal medium supplemented with yeast extract [2 g l^{-1}]. The used carbon source was in form of different ratios of xylose and xylan to give a final carbohydrate concentration of 80 g l^{-1} . **Fig. 4.53** shows the results of cell growth and enzyme production after 48, 96 and 144 h of cultivation in different media.

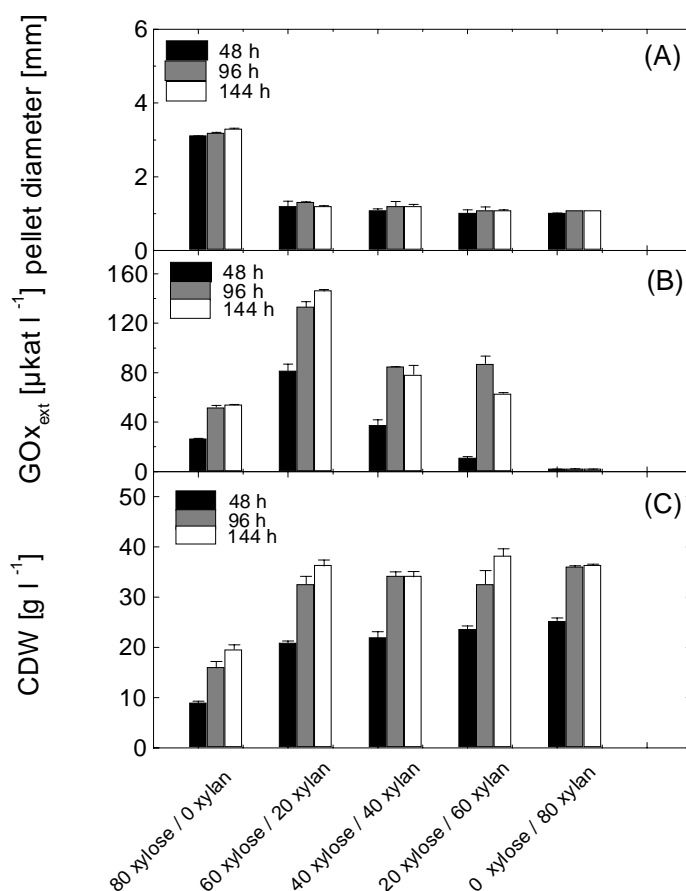


Figure 4.53: Cell growth and GOx production during a recombinant *A. niger* cultivation on different ratios (xylose:xylan) in shake flask culture.

The cell dry weight in case of mixed substrate culture (MSC) in all ratios under study was at least 50% higher compared to pure xylose culture. The increase in cell mass was due to the form of growth. In all cultivations other than pure xylose culture, the growth was mainly in form of small pellets with a small fraction of dispersed mycelial growth. The presence of xylan in the cultivation medium inhibited spore aggregation during the early time which resulted in a smaller pellet formation. Consequently the mass transfer limitation in these cultures was less leading to a significant increase in substrate consumption and cell mass formation.

In order to express the relation between GOx production and cell concentration, a plot of specific enzyme activity versus medium composition has been presented in **Fig. 4.54**.

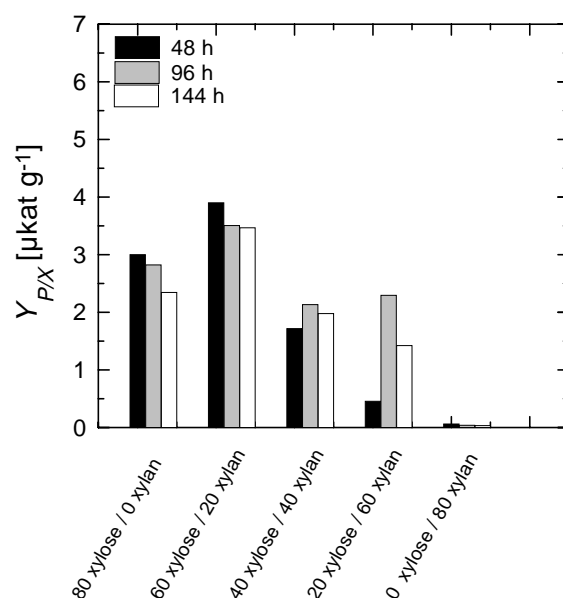


Figure 4.54: Glucose oxidase production yield as a function of different mixed substrate systems.

It is clearly observed that the increase in GOx production was mainly through the increase in biomass and the enhancement of morphological structure. The addition of a biopolymer such as xylan decreased the affinity of spores toward aggregation giving rise to a higher number of pellets with a reduced size. The active fraction of the biomass in these cultures, mainly the outer layer of pellet, became more than those of larger pellet leading to a better substrate consumption and GOx excretion [**Fig. 4.53**].

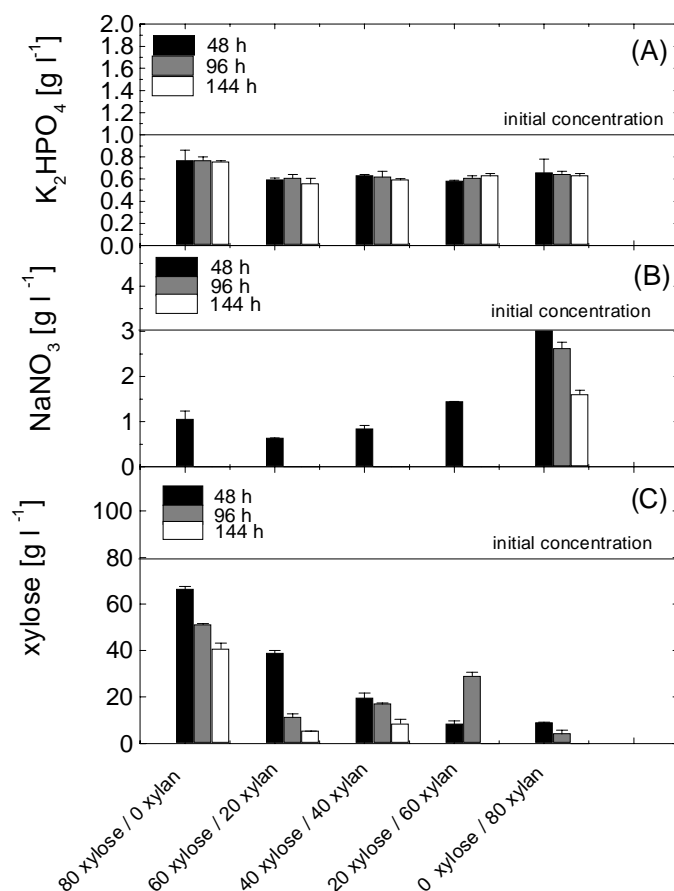


Figure 4.55: Consumption of different substrates during *A. niger* cultivation using mixed substrate (xylose/xylan) in shake flask culture.

Looking at other substrates in the cultivation medium such as N-source and P-source after 48 h of cultivation, the maximal consumption of sodium nitrate was observed in the culture using xylose/xylan mixture with a ratio of (3:1). This consumption was about 30% higher than using xylose as sole C-source. In case of using xylan as sole C-source, the nitrate consumption was very low and increased with the time and more than 50% of the initial nitrate concentration remained non-utilized after 144 h of cultivation. On the other hand, a complete consumption of nitrate was observed in all mixed substrate cultures as well as in pure xylose culture after 96h [Fig. 4.55]. The consumption of phosphate in all MSC and the pure xylan culture was almost the same during the cultivation which was about 15% higher compared to pure xylose culture.

However, the effect of addition of either insoluble particles or a biopolymer to the cultivation medium on cell morphology in submerged culture of filamentous microorganisms was studied by

other authors (Byrne and Ward, 1987). They found that the addition of biopolymers to the cultivation medium converted the growth of *Rhizopus arrhizus* from pellet form to dispersed growth form. The enhancement of dispersed growth with addition of biopolymer may occur by physical separation of spores and mycelia, preventing attraction of spores and mycelial, and aggregation as well (Metz and Kossen, 1977). By this way, a prohibition of a large spore aggregate was observed and subsequently the growth resulted in form of dispersed mycelium and micropellets minimizing the mass transfer limitation inside the bioparticles. From our previous results we can conclude that, the addition of xylan to cultivation medium improved GOx production mainly through the improvement of cell morphology (formation of high number of pellet with small diameter). Since the double substrate cultivation for a xylose-xylan ratio 3:1 remained most efficient for GOx production, a further cultivation was conducted in a stirred tank bioreactor with this ratio.

4.7.2. Batch cultivation in xylose/xylan culture in 5-L bioreactor

4.7.2.1. Cell growth, glucose oxidase production, substrate consumption and acid production

Based on our previous results, a batch cultivation using a xylose:xylan mixture ($60 \text{ g l}^{-1} : 20 \text{ g l}^{-1}$) was carried out in 5-L bioreactor. After a lag time of about 5 h, spores germinated giving rise to a short germ tube. The fungus apparently grew exponentially and reached a maximum of 16 g l^{-1} CDW after 35 h and kept more or less constant for the rest of cultivation time [Fig. 4.56 and Tab. 9.1.6, Appendix].

The growth was mainly of mycelial form with micropellets of smaller diameter than those obtained in culture without xylan. Compared to pure xylose cultures, the consumption of nitrate and phosphate was higher with only a little differences in xylose consumption during the exponential growth phase. The nitrate was almost totally consumed after 30 h. However, the quantitative determination of xylose consumption might not be highly accurate, especially at the late time of cultivation, due to the presence of xylan which might be decomposed to xylose.

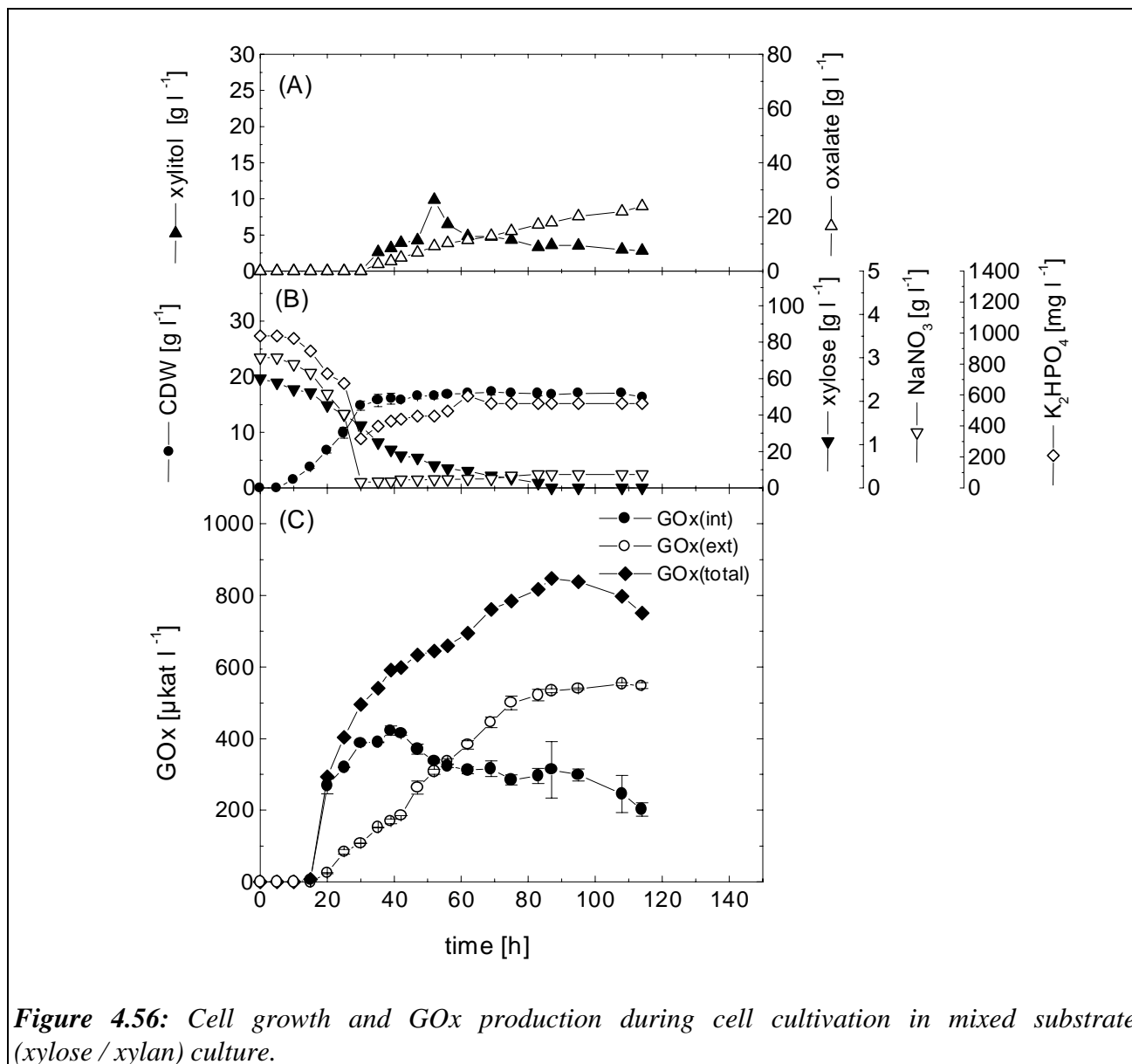


Figure 4.56: Cell growth and GOx production during cell cultivation in mixed substrate (xylose / xylan) culture.

The addition of xylan showed no significant effect on the germination time and the germ tubes emerged from the spore body 5-6 h after incubation. The volumetric enzyme production increased with the time reaching about 850 $\mu\text{kat l}^{-1}$ after 87 h of cultivation (about 38% higher than the value of volumetric GOx produced in batch culture using pure xylose 80 g l⁻¹). The rate of GOx production reached to a maximum of 57.3 $\mu\text{kat l}^{-1} \text{ h}^{-1}$ with a specific productivity of about 8 $\mu\text{kat h}^{-1} \text{ g}^{-1}$ after 20 h [Fig. 4.57].

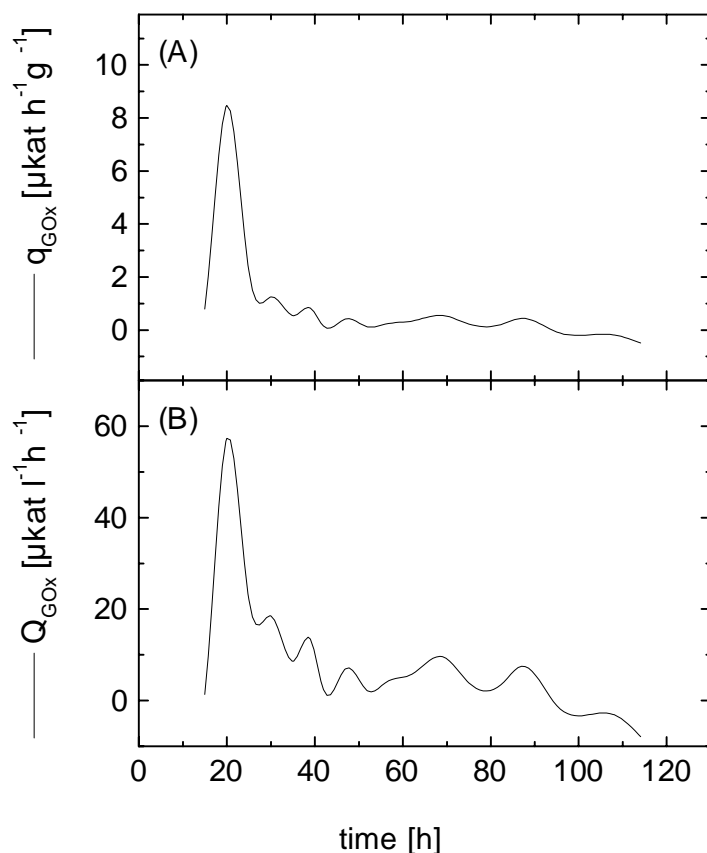


Figure 4.57: Volumetric and specific GOx production rates during a recombinant *A. niger* cultivation in mixed substrate (xylose/xylan) culture.

Xylitol was also formed in this culture as intermediate product reaching to a maximum of about 10 g l^{-1} and was consumed again for oxalic acid production. The oxalate was first detected in this culture after 35 h and increased with the time reaching about 22 g l^{-1} at the end of cultivation. The oxalate produced was less compared to pure xylose culture under the same cultivation conditions.

The beneficial effects of soluble polymers might be reflected in high permissible power inputs or tip speed in the bioreactor since these polymers could depress local velocity gradients close to the impeller thus preventing cell damage (Moo-Young *et al.*, 1969). Moreover, growth possibly has been stimulated by polymers enhancing the mass transfer of nutrients to the mycelia (Moo-Young *et al.*, 1969; Elmayergi and Moo-Young, 1973). In the present work xylan increased the growth rate during the early time of cultivation. After that time the biomass concentration was almost the same as in the case of pure xylose culture due to the limitation in N-source after 35 h.

4.7.2.2. Carbon balance

Calculation of carbon balance was carried out using **Eq. 4.3**, neglecting the participation of xylan. As observed in **Fig. 4.58** the carbon recovery was near to 100% during the first 30 h. After this time, the carbon recovery was more than 100% which indirectly indicated the degradation of xylan in this culture. However, it was not possible to quantify the degradation of xylan during this cultivation due to the presence of xylose (the mono-sugar of xylan) in high concentrations.

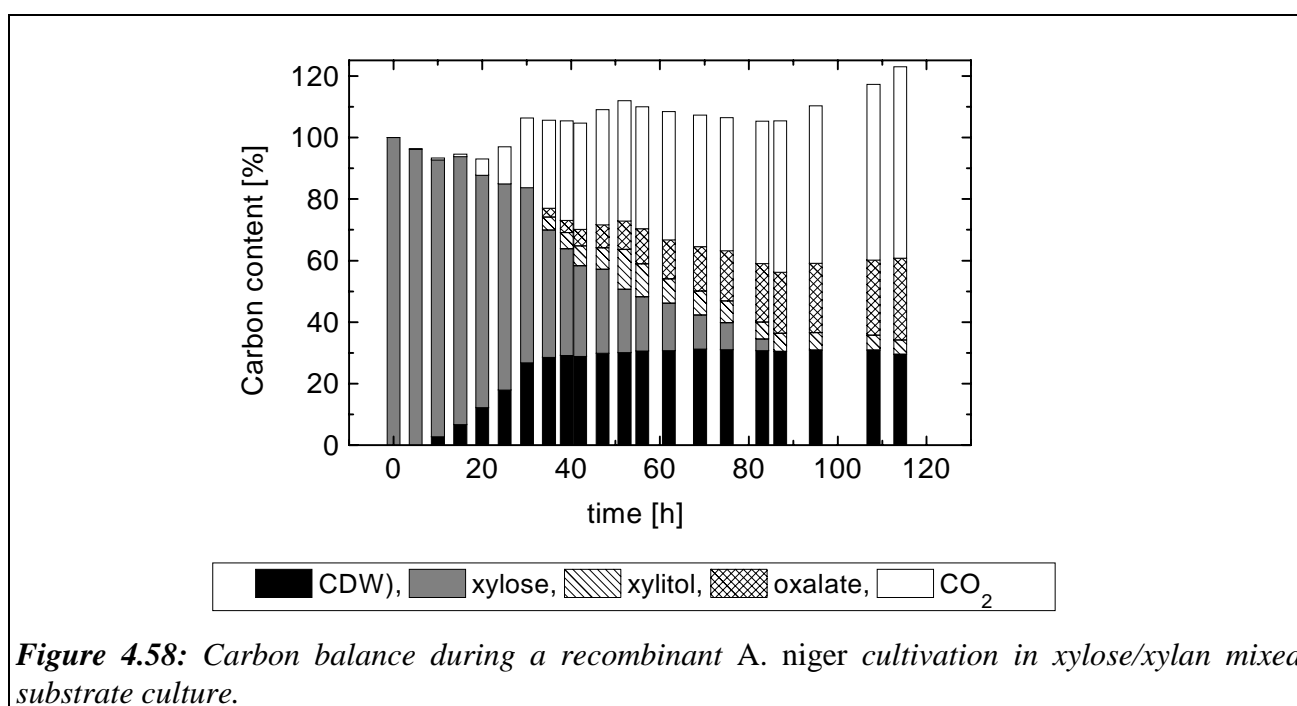


Figure 4.58: Carbon balance during a recombinant *A. niger* cultivation in xylose/xylan mixed substrate culture.

4.8 Comparison between pure xylose and xylose-xylan culture at bioreactor level

A direct comparison between cell growth, GOx production and excretion in pure xylose and xylose/xylan cultures are represented in **Fig. 4.59**. It is observed that the addition of xylan enhanced cell growth at the early phase of cultivation reaching to a maximum of about 16 g l⁻¹ after about 30 h (10 hours earlier than pure xylose culture). On the other hand, both the amounts of enzyme production and excretion during this growth phase were more or less the same in both cultures. This may be due to the fact that no big differences in growth form and that the whole biomass was considered as an active fraction. After this time the increase in extracellular enzyme concentration in case of mixed substrate culture was through further enzyme production [**Figs. 4.59, A and B**].

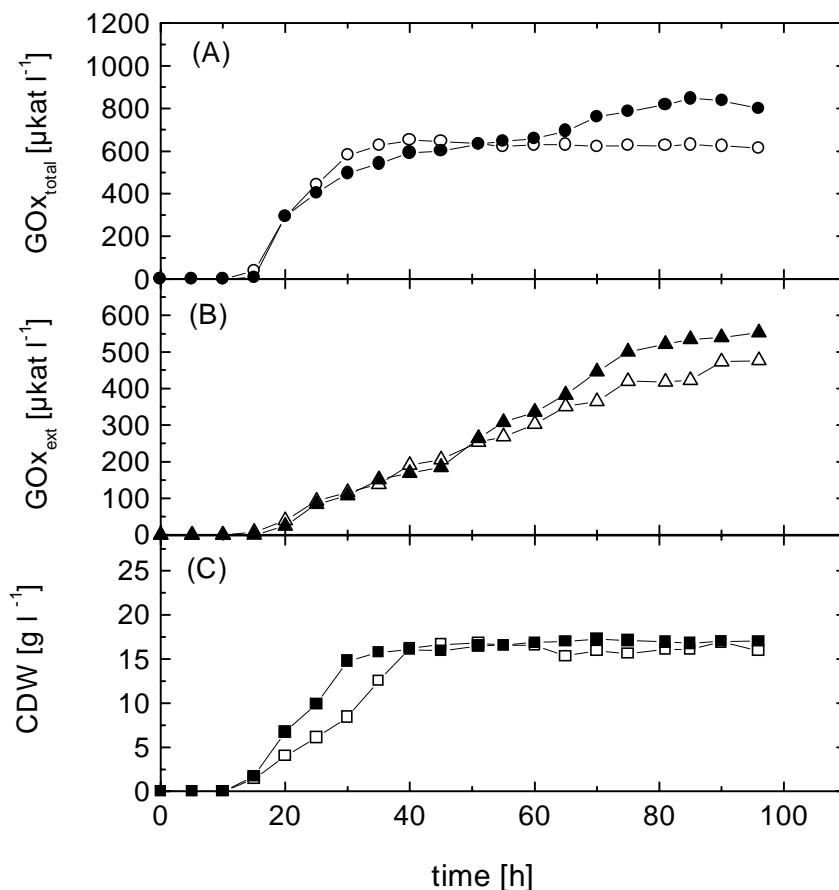


Figure 4.59: Comparison between cell growth and GOx production in pure xylose culture and mixed substrate cultures. Open and close symbols refer to pure xylose (80 g l^{-1}) and xylose/xylan ($60/20 \text{ g l}^{-1}$) cultures, respectively.

The addition of biopolymer like xylan to the medium supported GOx production for longer time as a culture under less hydrodynamic stress (500 rpm agitated culture). Therefore we consider that xylan functioned as protectant for the mycelium from hydrodynamic stress which caused by agitation. This phenomena is supported also by the calculation of the oxygen consumption rate for these cultures [Fig. 4.60].

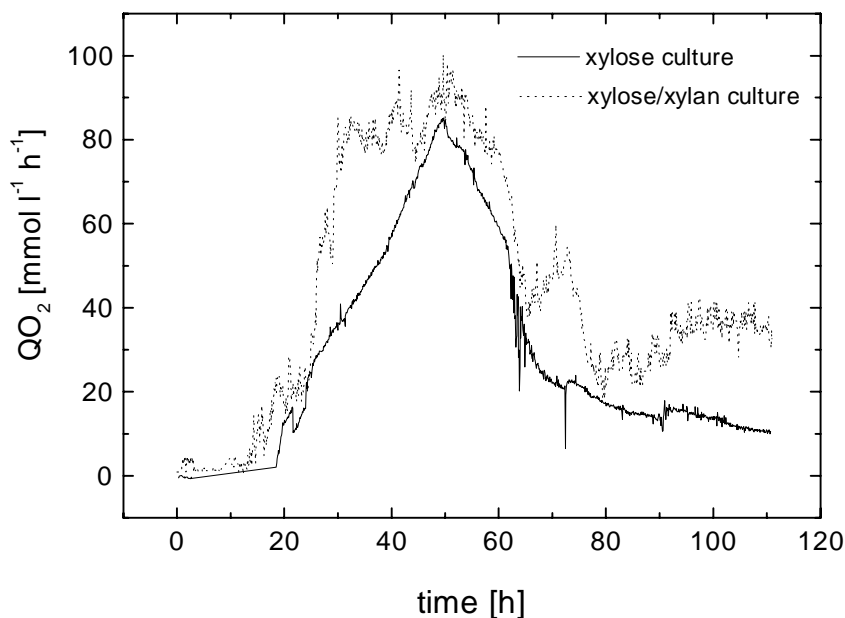


Figure 4.60: Oxygen consumption rate during a recombinant *A. niger* cultivation in pure xylose- and mixed substrate-cultures.

In spite of the same biomass in both pure xylose and xylose/xylan cultures, the rate of oxygen consumption in the later culture was higher which indirectly indicated the higher activity of cells. These results suggested the protective effect of xylan indirectly exerted some biological effects concerning GOx production.

The main advantages of mixed substrate (xylose/xylan) were:

- 1- The volumetric production of GOx was increased by about 38%.
- 2- The production yield of enzyme was also increased by about 35%.
- 3- The formation of the undesirable organic acids such as oxalic acid was decreased.

4.9 Fed-Batch cultivation of a recombinant *Aspergillus niger* in a 5-L bioreactor

The aim of this experiment was to increase both the cell mass and the GOx production during cultivation at agitation speed of 800 rpm through an exponential feeding of the utilized substrates. A simple fed-batch process was performed to obtain a high cell density culture according to the method of Korz *et al.* (1992). After a batch-phase of 33 h, the fed-batch-phase was started, and for a desired specific growth rate [μ] and a given biomass concentration [X_F] the actual feed rate of substrates was calculated as follows:

$$m_s(t) = \left[\frac{1}{Y_{X/S}} \cdot \mu_{set} + m_E \right] \cdot V_L(t) \cdot X_F \cdot e^{\mu_{set}(t-t_F)} \quad [4.6]$$

where

m_s	Mass flow of substrate [g h^{-1}]
t	Cultivation time [h]
t_F	Start time of feeding phase [h]
μ	Specific growth rate [h^{-1}]
m_E	Maintenance coefficient [$\text{g g}^{-1} \text{h}^{-1}$]
$Y_{X/S}$	Yield coefficient, biomass/substrate [g g^{-1}]
X_F	Biomass concentration at the start time of feeding phase [g]
V_L	Culture volume [L]

The following results were obtained from the batch cultivation (800 rpm):

t_F	33 h
μ_{set}	0.08 h^{-1}
m_E	assumed to be zero
$Y_{X/xylose}$	0.5 g g^{-1}
$Y_{X/nitrate}$	7.0 g g^{-1}
X_F	15 g
V_L	3 L

The cultivation was started using the normal minimal medium supplemented with yeast extract [2 g l^{-1}] and xylose [40 g l^{-1}]. The cultivation conditions and inoculum preparation were the same as

in the batch culture. After the initial batch phase, the fed-batch phase was started (after 34 h) using a pre-determined exponential feeding rate according to **Eq. 4.4** to maintain the constant specific growth rate of $0.08 \text{ [h}^{-1}\text{]}$. The feeding solution was composed of the following $[\text{g l}^{-1}]$: xylose, 200.0; NaNO_3 , 57.0; K_2HPO_4 , 19; KCl , 8.5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2.

The rate of substrate feeding was started with 0.50 ml min^{-1} and increased exponentially with the time [**Fig 4.61**].

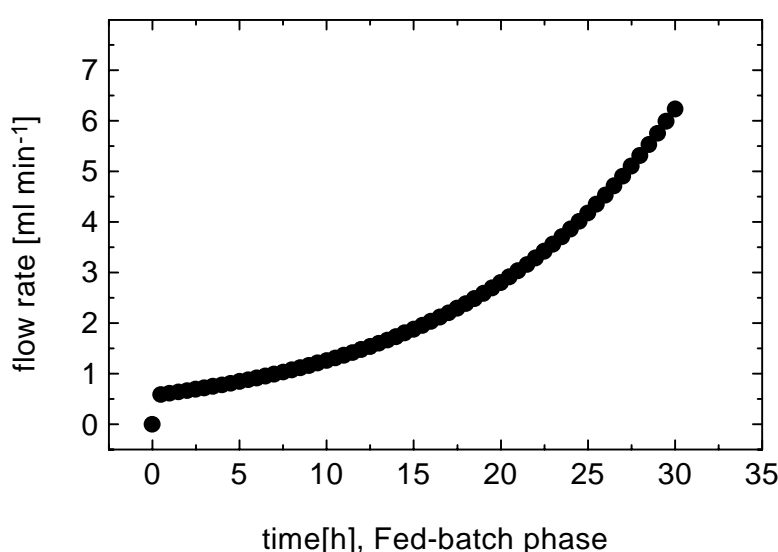


Figure 4.61: Rate of substrate feeding during fed-batch cultivation.

4.9.1 Cell growth and substrate consumption

The germination of fungal spores started after a lag phase of about 7 h. The fraction of germinating spores was less than those in the batch culture at the same agitation speed. This may be due to the lower initial xylose concentration. The macro-morphological growth during the first 33 h of cultivation (batch phase) was mainly of mycelial form with a few micro-pellets. As feeding started, cells grew extensively with the same morphological features. As feeding was terminated after 55 h, the macro-morphological structure slowly converted from mycelial form to pellet form. The results of cell growth, oxygen consumption and carbon dioxide production during controlled feeding are represented in **Fig. 4.62**

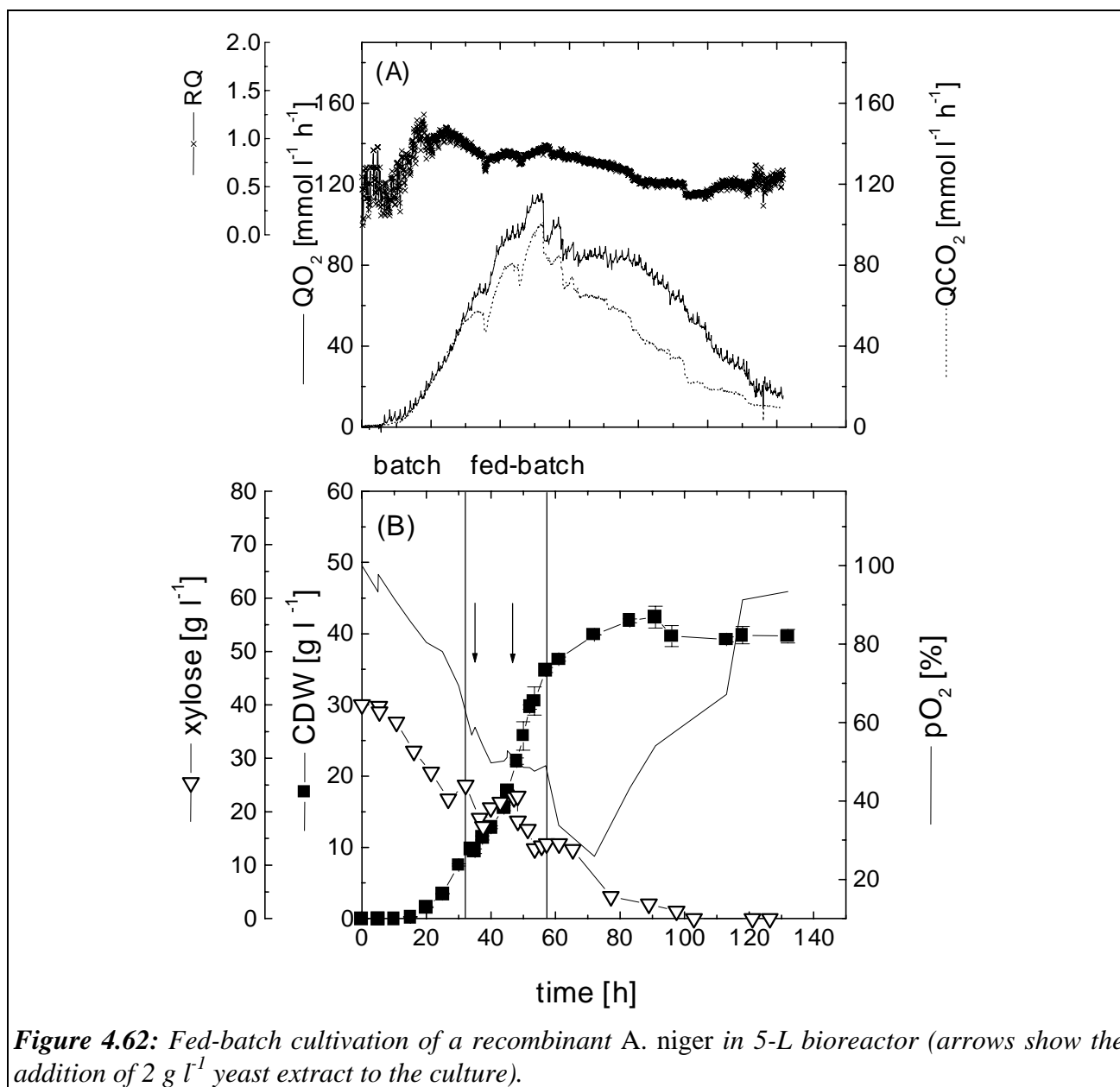
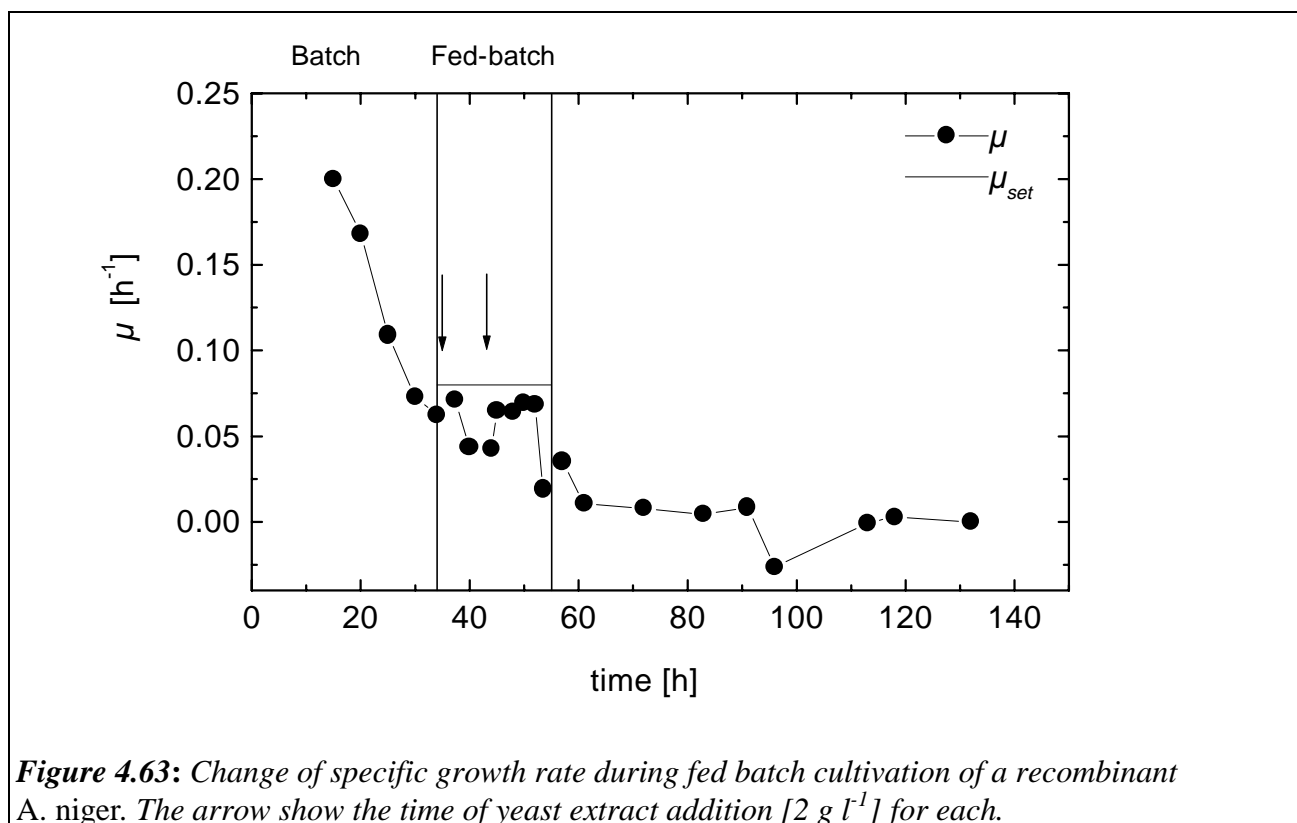


Figure 4.62: Fed-batch cultivation of a recombinant *A. niger* in 5-L bioreactor (arrows show the addition of 2 g l^{-1} yeast extract to the culture).

As the feeding started, the oxygen consumption rate kept more or less constant for 1 hour, which indicated indirectly the termination of growth. Therefore, yeast extract was added with a concentration of 2 g l^{-1} at 35 h. Again at 45 h yeast extract was added to support the growth in this culture. After yeast extract addition in both cases, a significant consumption of xylose was observed with a concomitant increase of both oxygen consumption- and carbon dioxide production rates. During the feeding time, the cell dry mass increased from 10 g l^{-1} up to about 33 g l^{-1} . However, at the beginning of the feeding, the specific growth rate was lower than the set value. The decrease of the specific growth rate was recovered after the addition of yeast extract [Fig. 4.63].



As shown previously, an addition of yeast extract was necessary for the continuation of further cell growth in the fed-batch phase. Therefore this result indicates that even in non C- and N- limited conditions yeast extract is necessary to support cell growth.

4.9.2 Glucose oxidase production and excretion

The production curves of GOx by a recombinant *A. niger* in fed-batch culture are presented in **Fig. 4.64**. During the first batch phase, the volumetric extracellular GOx activity increased from zero (15 h) to about 53 $\mu\text{kat l}^{-1}$ (34 h). During this phase, GOx was accumulated in the cells reaching 397 $\mu\text{kat l}^{-1}$ (34 h). The GOx production rate was 11.26 $\mu\text{kat l}^{-1} \text{h}^{-1}$. As the feeding started, the rate of enzyme production increased significantly and reached 44.75 $\mu\text{kat l}^{-1} \text{h}^{-1}$ (as a sum of extra- and intracellular activities). The increase in GOx production rate after the termination of feeding was low, only about 5.78 $\mu\text{kat l}^{-1} \text{h}^{-1}$.

On the other hand, the enzyme excretion increased with the time. The enzyme excretion rates were 1.31; 12.30 and 14.97 $\mu\text{kat l}^{-1} \text{h}^{-1}$ during the early batch phase, feeding phase and post feeding phase, respectively.

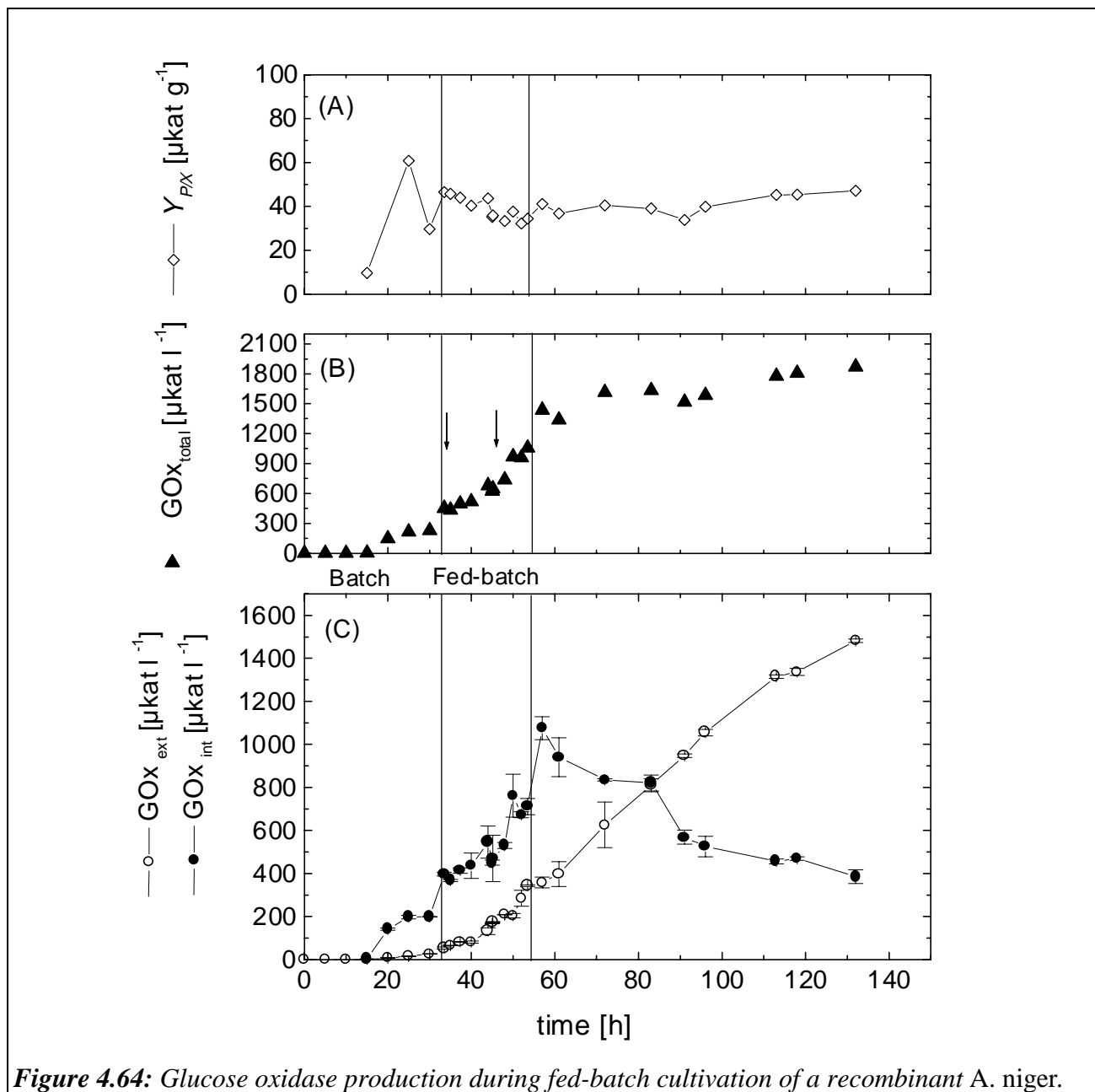


Figure 4.64: Glucose oxidase production during fed-batch cultivation of a recombinant *A. niger*.

Since GOx is a cell associated primary metabolite, the increase in cell mass resulted in a higher production of the enzyme. Moreover, the cell growth during the production phase was of micropellet-filamentous form (the suitable growth form for GOx production). Thus, the specific yield of enzyme produced [$Y_{P/X}$] was predicted to be equal to a batch grown culture under the same agitation speed. With the exception of the early cultivation phase, the specific yield of the enzyme production was of about $40 \pm 4 \mu\text{kat g}^{-1}$. This yield was more or less equal to the value obtained

from batch cultures under the same agitation speed. The increase of the volumetric enzyme production in this culture was mainly through the increase in cell mass.

A little information is available regarding the cultivation of filamentous microorganisms in fed-batch cultures for high cell density. The difficulties of fungal cell cultivation in high cell density cultures are mainly attributed to the mode of growth compared to unicellular microorganisms. While the growth form is a critical factor in this process as described previously, not only a high cell mass is required but also the growth in the desired morphology. Kerns *et al.* (1987) cultivated *A. niger* in fed-batch cultures for β -glucosidase production. They found that the volumetric enzyme production increased up to 5 folds with intermittent glucose addition but they did not give details about the influence of feeding on the increase of cell mass. Also, Chen, (1993) studied the fed-batch cultivation of *Aspergillus foetidus* for citric acid production under phosphate and nitrogen limited conditions. The maximal cell dry weight of 28 g l^{-1} was obtained after 16 days cultivation. Imai *et al.* (1994) studied the cultivation of *A. oryzae* for glucoamylase production in fed-batch culture. They increased the cell mass up to 33 g l^{-1} accompanied with an enzyme production of about 0.875 U ml^{-1} after 120 h cultivation in a stirred tank bioreactor. In all these previous studies no details were given about either growth morphology or cell productivity compared to the corresponding batch culture.

In our study, the increase in cell mass up to 45 g l^{-1} CDW in the desired morphological growth form was achieved resulting in the GOx overproduction reaching about $1800 \text{ } \mu\text{kat l}^{-1}$. The cell productivity in fed-batch culture was almost the same as in the case of batch one, cultivated with the same type of inoculum and under the same agitation speed. Thus, this result supports also the tight relation between the growth morphology and the cell productivity as mentioned above (see 4.6.3).

5. Summary

The interrelation between various process parameters was evaluated for the cultivation of a recombinant *Aspergillus niger* NRRL 3 (GOD 3-18) in shake flask and bioreactor level. To increase GOx production in submerged culture, yeast extract was added to cultivation medium up to a concentration of 2 g l⁻¹. Beyond this concentration yeast extract supported only cell growth with a decrease in enzyme production due to the formation of large pellets. For further study of the influence of growth morphology on cell productivity, cultivations in baffled and non-baffled flask were performed. In the case of baffled flasks, cells grew in smaller pellet form which resulted in higher productivity as compared to non-baffled flask. The process was then conducted at bioreactor level for GOx production under controlled pH value. The origin of inoculum was a critical factor in microbial growth. When the spores, obtained directly from stock frozen glycerin culture, were plated only once the growth resulted in micropellet-filamentous form, showing more than two fold increase in GOx production and with a higher excretion rate as compared to pelleted grown cultures. The pelleted growth was observed under the same cultivation conditions when spores were obtained from multi-time plated cultures.

The second part of this study was focused on the production of GOx with non-glucose C-sources to overcome the problem of the conversion of the substrate glucose by the produced enzyme. Among nine different mono-sugars tested, fructose and xylose supported cell growth and GOx production. Utilization of fructose increased the final cell mass concentration 2-fold compared to glucose at equivalent concentration, but it resulted in 30% lower final total GOx activity. On the other hand, xylose increased the final cell mass concentration and the final total GOx activity were 3 and 2.5-fold higher, respectively. The suitability of fructose and xylose for GOx production was also studied in a 5-L bioreactor. The reduced enzyme production in case of fructose culture was attributed to the growth in pellets which decreased the GOx production and excretion. On the other hand, growth in xylose culture was in the optimal growth form (micropellet-filamentous form) with a higher GOx production and cell mass.

In the third part of this work, the dependency of production and excretion of GOx from recombinant *A. niger* on the morphological growth in xylose culture was investigated. Cultivations were carried out in 5-L bioreactor under different agitation speeds at 200, 500 and 800 rpm. During these cultivations, significant differences in fungal growth were observed. During cultivation under a high agitation speed, *A. niger* grew in micropellet form embedded in a filamentous network. In less agitated culture, the fraction of filamentous growth decreased and the diameter of aggregates increased concomitantly. The relation between these differences in growth and the amount of the produced enzyme was studied. The growth in micropellet-filamentous form was the most suitable for enzyme production. After 40 hours, the enzyme production reached values of about 650 μkat , 380 μkat and 220 $\mu\text{kat l}^{-1}$ in 800, 500 and 200 rpm agitated cultures, respectively.

Looking at the excreted enzyme fraction, a significant difference was also observed between these cultures. During the first 40 hours of cultivation, the average excretion rates of the enzyme were 2.37, 5.14 and 7.08 $\mu\text{kat l}^{-1} \text{ h}^{-1}$ in 200, 500 and 800 rpm agitated cultures, respectively.

The studies of growth morphology using fluorescence microscope after staining of the bioparticle with acridine orange (AO) showed that the active growing part was restricted to the outer layer of the pellet. In case of filamentous-micropellet grown culture, the whole biomass appeared active. On the other hand, in the case of pelleted growth, only the outer layer of the pellet with a thickness 100-150 μm was active. The thickness of this layer was dependent on the agitation speed and independent of the pellet size. As the pellet size increased the inactive fraction of biomass increased as well.

This study showed that neither high nor low agitation speeds were suitable for GOx production for two different reasons. In the former case the high agitation resulted in lower cell mass and growth terminated after 40 h due to a high shear stress. On the other hand, at low agitation speed growth was mainly in large pellet form resulting in a lower enzyme production and excretion. The maximal volumetric enzyme production was obtained using an intermediate agitation of 500 rpm which gave a better cell growth with a moderate size pellet.

Further improvement of extracellular GOx production was achieved by manipulation of the fungal morphology by addition of an organic polymer such as xylan to the culture broth. In shake flask cultures, the addition of xylan resulted in smaller pellets of higher number, increasing the active fraction of biomass in these cultures. This morphological change resulted in an increase of about 2.5-fold in extracellular GOx production compared to pure xylose culture. In the stirred tank bioreactor, the increase in GOx production was only about 35% since no significant difference in growth morphology was observed in both cultures and since xylan worked as protectant against cell breakdown under a high agitation speed.

Further improvement in GOx production was achieved by increasing the cell mass, in the optimal growth form (micropellet-filamentous form), using a fed-batch cultivation strategy. Using this method of cultivation, an increase in cell mass up to 45 g l^{-1} was achieved with a concomitant increase in enzyme production reaching $1800 \mu\text{kat l}^{-1}$.

In conclusion, the production of GOx controlled by the *gpdA* promoter is not dependent on the presence of glucose. Improvement of GOx production was achieved either by employment of pentose sugar (xylose), changing of the morphology to the desired form through the type of inoculum and agitation speed or by using xylose/xylan mixture as C-source. Further improvement of GOx production was also achieved through the increase of cell mass (in the desired growth form) in fed-batch culture. Moreover, with the knowledge of the thickness of the actively growing layer of the fungal pellet and the distribution of pellet population a mathematical relation between cell yield and morphology was developed.

6. Zusammenfassung

Die Beziehung zwischen verschiedenen Prozeßparametern wurde für die Kultivierung von rekombinanten *Aspergillus niger* NRRL 3 (GOD 3-18) in Schüttelkultur und Bioreaktorexperimenten untersucht. Um die GOx Produktion in Submerskultur zu steigern, wurde Hefeextrakt zum Kulturmedium bis zu einer Konzentration von 2 g l⁻¹ hinzugefügt. Oberhalb dieser Konzentration führte Hefeextrakt nur zu Zellwachstum bei gleichzeitiger Abnahme der Enzymproduktion, da größere Pellets gebildet wurden. Zur weiteren Untersuchung des Einflusses der Morphologie auf das Zellwachstum wurden Kultivierungen in Schüttelkolben mit und ohne Schikanen durchgeführt. In Kolben mit Schikanen wuchsen die Zellen in kleineren Pellets, was zu höherer Enzymproduktivität im Vergleich zu Kolben ohne Schikanen führte. Daraufhin wurde der Prozeß in einem Bioreaktor mit geregelter pH durchgeführt.

Der Ursprung des Inokulum war ein kritischer Faktor für das mikrobielle Wachstum. Wenn die Sporen, von einer Glycerinstammkultur stammend, nur einmal ausplattiert wurden, zeigte sich filamentöses Wachstum mit geringem Anteil von Mikropellets. Gleichzeitig verdoppelte sich die GOx Produktion und auch die Exkretion stieg an, im Vergleich zu Kultivierungen in Pelletform. Wachstum in Pelletform wurde beobachtet unter ansonsten den gleichen Bedingungen, wenn Sporen von mehrfach ausplattierten Kulturen genommen wurden.

Im zweiten Teil dieser Arbeit wurde die Produktion von GOx mit verschiedenen Kohlenstoffquellen, außer Glukose, untersucht, um den Umsatz von Glukose durch das produzierte Enzym zu vermeiden. Von den neun getesteten Mono-sacchariden unterstützten Fruktose und Xylose Zellwachstum und GOx Produktion. Die Aufnahme von Fruktose steigerte die Zellmasse 2-fach im Vergleich zu Glucose bei äquivalenter Konzentration, aber die Gesamt-GOx-Aktivität war 30% niedriger. Auf der anderen Seite steigerte Xylose die Biomassekonzentration 3-fach und die Gesamt-GOx-Aktivität 2.5-fach. Kultivierungen mit Fruktose und Xylose wurden auch in einem 5-L-Bioreaktor durchgeführt. Die reduzierte Enzymproduktion im Fall der Kultur mit Fruktose kann wahrscheinlich auf das Wachstum in Pellets zurückgeführt werden. Das Wachstum in der Xylose-Kultur fand in der optimalen Wachstumsform (filamentös, mit Mikropellets) statt: Die Biomasse und GOx-Produktion waren entsprechend höher.

Im dritten Teil dieser Arbeit wurde die Abhängigkeit der GOx-Produktion und Exkretion durch rekombinanten *A. niger* von der Morphologie in Kultivierungen mit Xylose als C-Quelle untersucht. Die Kultivierungen wurden in einem 5-L-Bioreaktor bei unterschiedlichen Rührgeschwindigkeiten (200, 500 und 800 U/min) durchgeführt. Signifikante Unterschiede im Pilzwachstum wurden beobachtet. Kultivierungen bei höherer Drehzahl zeigten Wachstum in Mikropellets, die in ein filamentöses Netzwerk eingebettet waren. Bei niedrigeren Drehzahlen nahm das filamentöse Wachstum ab und der Durchmesser der Aggregate stieg gleichzeitig. Der Zusammenhang zwischen Morphologie und Enzymproduktion wurde untersucht. Höchste Enzymproduktion wurde bei Wachstum in Mikropelletform gefunden. Nach 40 Stunden erreichte die Enzymaktivität Werte von ca. 650, 380 und 220 $\mu\text{kat l}^{-1}$, für die Kulturen bei jeweils 800, 500 und 200 U/min.

Ein weiterer Unterschied bei diesen Kulturen wurde hinsichtlich des exkretierten Enzymanteils beobachtet. Für die Kultivierungen bei 200, 500 und 800 U/min wurden jeweils Enzymexkretionsraten von 2.37, 5.14 und 7.08 $\mu\text{kat l}^{-1}\text{h}^{-1}$ während der ersten 40 Stunden gemessen.

Die Morphologie von *Aspergillus niger* wurde untersucht, indem die Biopartikel mit Acridinorange (AO) gefärbt wurden. Anschließend wurden sie mit Fluoreszenzmikroskopie detektiert und quantifiziert. Es zeigte sich, daß nur die äußere Schicht des Pellets aktiv war. Im Falle des Mikropelletwachstums erschien die ganze Biomasse aktiv. Im Falle des Pelletwachstums war nur die äußere Schicht, etwa 100 - 150 μm dick, aktiv. Die Schichtdicke war abhängig von der Rührerdrehzahl, aber unabhängig von der Pelletgröße. Mit zunehmender Pelletgröße stieg der inaktive Teil der Biomasse ebenfalls an.

Die Untersuchungen zeigten, daß weder hohe noch niedrige Rührerdrehzahlen geeignet sind für die GOx-Produktion, und zwar aus folgenden zwei Gründen:

- 1- Bei hohen Drehzahlen war die Zellmasse niedriger, und das Wachstum endete nach 40 Stunden wegen hohen Scherstress.
- 2- Bei niedrigen Drehzahlen war das Wachstum in Form von großen Pellets, was niedrigere Enzymproduktion und Exkretion zur Folge hatte.

Die maximale volumetrische Enzymproduktion wurde bei einer mittleren Drehzahl von 500 U/min erhalten, bei der besseres Wachstum mit kleiner Pelletgröße erreicht wurde.

Weitere Verbesserung der GOx Produktion wurde durch Änderung der Pilzmorphologie erreicht, in dem ein organisches Polymer, z.B. Xylan, zu den Kulturen hinzugefügt wurde.

In Schüttelkolben, resultierte die Zugabe von Xylan in kleineren aber mehr Pellets, was den Anteil der aktiven Biomasse in diesen Kulturen erhöhte. Die morphologische Veränderung hatte einen 2.5-fachen Anstieg der extrazellulären GOx-Produktion zu Folge, im Vergleich zur Kultivierung ohne Xylan. Bei Kultivierungen im Bioreaktor war der Anstieg der GOx-Produktion nur ca. 35%, da beides Kultivierungen ähnliche Morphologien aufwiesen und da Xylan schützend gegen Zellfragmentierung, die bei hohen Drehzahlen auftreten kann, wirkt.

Weitere Verbesserung der GOx Produktion wurde durch Erhöhung der Biomassekonzentration in optimaler Wachstumsform (Mikropellet-filamentös) im fed-batch Betrieb erreicht. Durch Anwendung dieser Kultivierungsstrategie wurde eine Biomassekonzentration bis zu 45 g l^{-1} bei gleichzeitiger Erhöhung der Enzymproduktion ($1800 \text{ } \mu\text{kat l}^{-1}$) erreicht.

Schlußfolgernd, läßt sich sagen, daß die Produktion von GOx, die unter der Kontrolle des *gpdA* Promotor ist, unabhängig vom Vorhandensein von Glucose ist. Verbesserung der GOx Produktion wurde durch einen Pentosezucker (Xylose) als C-Quelle erreicht, durch die Veränderung der Morphologie durch die Art der Inokulum- vorbereitung bzw. durch die Drehzahl, und durch ein Xylose/Xylan Gemisch als C-Quelle. Weitere Verbesserung der GOx-Produktion wurde durch die Erhöhung der Biomassekonzentration in fed-batch-Kultur erreicht. Darüberhinaus, da die aktive Schicht des Pilzpellets und die Verteilung der Pelletgröße gewessen wurde, konnte eine mathematische Beziehung zwischen Biomasseausbeute und Morphologie entwickelt werden.

7. Symbols and abbreviations

7.1 Abbreviations

ABTS		2,2'-Azino-di-[3-ethylbenzthiazolin sulfonate]
<i>A. niger</i>		<i>Aspergillus niger</i>
<i>A. nidulans</i>		<i>Aspergillus nidulans</i>
AO		Acridine Orange
ATP		Adenosin triphosphate
CDW	[g l ⁻¹]	Cell Dry Weight
d_i	[mm]	Impeller diameter
d_t	[mm]	Tank diameter
DNA		Deoxy ribonucleic acid
DNS		3,5- Dinitro salicylic acid
Eq.		Equation
ER		Endoplasmic reticulum
FAD		Flavin adenine dinucleotide
FDA		Food and Drug administration
Fig.		Figure
GOx		Glucose oxidase
GOx _{int}	[μkat l ⁻¹]	Intracellular glucose oxidase
GOx _{ext}	[μkat l ⁻¹]	Extracellular glucose oxidase
GOx _{total}	[μkat l ⁻¹]	Total glucose oxidase [GOx _{int} + GOx _{ext}]
GRAS		Generally Regarded As Safe
HPLC		High Performance Liquid Chromatography
MSC		Mixed Substrate Culture
<i>N. crassa</i>		<i>Neurospora crassa</i>
<i>P. chrysogenum</i>		<i>Penicillium chrysogenum</i>
<i>P. variable</i>		<i>Penicillium variable</i>
pO ₂	[%]	soluble oxygen concentration
Q _{GOx}	[μkat l ⁻¹ h ⁻¹]	Volumetric glucose oxidase production rate

q_{GOx}	$[\mu\text{kat g}^{-1} \text{ h}^{-1}]$	Specific glucose oxidase production rate on cell basis
Q_{NaOH}	$[\text{mMol l}^{-1} \text{ h}^{-1}]$	Volumetric sodium hydroxide consumption rate
$Q_{\text{gluconate}}$	$[\text{g l}^{-1} \text{ h}^{-1}]$	Volumetric gluconic acid production rate
Q_{oxalate}	$[\text{g l}^{-1} \text{ h}^{-1}]$	Volumetric oxalic acid production rate
RER		Rough Endoplasmic Reticulum
RNA		Ribonucleic acid
<i>S. cerevisiae</i>		<i>Saccharomyces cerevisiae</i>
t_o		Inoculation time
t	[h]	Time
T_{50}	[h]	The time at which 50% of the enzyme was excreted to the meidium.
Tab.		Table
<i>T. reesei</i>		<i>Trichoderma reesei</i>
UBICON		Universal Bio-Process Control System

7.2 Abbreviations and symbols in equations

A	$[\mu\text{kat l}^{-1}]$	Activity of glucose oxidase in the sample
dE/dt	$[\text{min}^{-1}]$	The time dependent increase in absorbance at 420 nm
D	$[\mu\text{m}]$	Diameter
D_{active}	$[\mu\text{m}]$	Active pellet diameter
D_{inactive}	$[\mu\text{m}]$	Inactive pellet diameter
D_{total}	$[\mu\text{m}]$	Total Pellet diameter
D_h	$[\mu\text{m}]$	The average hyphal diameter
D_s	$[\mu\text{m}]$	The average spore diameter
ε		Extinction coefficient of ABTS [®] at 420 nm [43.20 ml $\mu\text{mol}^{-1} \text{ cm}^{-1}$]
f		dilution factor of the sample

Gn	[mol]	gluconic acid
k		constant
I	[cm]	The path-length of the cuvette
$l_{h,av}$	[μm]	The average total hyphal length
M	[g]	Biomass
M_o	[g]	Initial biomass
m_E	[$\text{g g}^{-1} \text{h}^{-1}$]	maintenance coefficient
m_s	[g h^{-1}]	mass flow of substrate
μ	[h^{-1}]	specific growth rate
μ_h	[h^{-1}]	Specific hyphal extension rate
μ_{sv}	[h^{-1}]	Specific spore swelling rate [h^{-1}]
μ_{bp}	[h^{-1}]	Specific bioparticle extension rate [h^{-1}]
Ox	[mol]	Oxalic acid
ρ	[kg m^3]	density
Q_{O_2}	[$\text{mmol l}^{-1} \text{h}^{-1}$]	Volumetric oxygen uptake rate
Q_{CO_2}	[$\text{mmol l}^{-1} \text{h}^{-1}$]	Volumetric carbon dioxide production rate
w	[μm]	The width of active outer mycelial shell of pellet
π		constant
RQ		Respiratory quotient
p	[1 atm]	Working pressure
R		Gas constant [$8.2057 \cdot 10^5 \text{ l atm mmol}^{-1} \text{K}^{-1}$]
t	[h]	Time
t_F	[h]	Starting time of feeding phase
T	[K]	Absolute temperature
V	[μm^3]	Volume
V_{active}	[μm^3]	Pellet active volume [μm^3]
$V_{inactive}$	[μm^3]	Pellet inactive volume [μm^3]
V_{total}	[μm^3]	Total pellet volume [μm^3]
V_{bp}	[μm^3]	Total bioparticle volume = [$V_s + V_h$] [μm^3]
V_h	[μm^3]	Hyphal volume [μm^3]
V_G	[l h^{-1}]	Air flow

V_L	[l]	Culture volume
V_s	[μm^3]	Spore volume
X	[mol]	Cell Dry Weight
$x^e \text{CO}_2$	[mol mol ⁻¹]	Mole fraction of carbon dioxide in inlet air
$x^e \text{O}_2$	[mol mol ⁻¹]	Mole fraction of oxygen in inlet air
$x^a \text{CO}_2$	[mol mol ⁻¹]	Mole fraction of carbon dioxide in off gas
$x^a \text{O}_2$	[mol mol ⁻¹]	Mole fraction of oxygen in off gas
X_y	[g l ⁻¹]	Xylitol concentration
σ_{CO_2}	[%]	Carbon content of carbon dioxide
σ_s	[%]	Carbon content of substrate
σ_{gn}	[%]	Carbon content of gluconic acid
σ_{ox}	[%]	Carbon content of oxalic acid
X_F	[g]	Biomass concentration at the starting time of feeding.
$Y_{p/x}$	[$\mu\text{kat g}^{-1}$]	Yield coefficient of GOx production per biomass
$Y_{x/s}$	[g g ⁻¹]	Yield coefficient, biomass/substrate

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9. Appendix

9.1. Cell growth, glucose oxidase production, substrate consumption and acid production during cultivation in 5-L bioreactor

Table 9.1.3a: Batch cultivation of a recombinant *A. niger* in 5-L bioreactor using glucose as a sole C-source (growth in pellet form).

Time [h]	CDW [g l ⁻¹]	GOx _{ext} [μkat l ⁻¹]	GOx _{int} [μkat l ⁻¹]	GOx _{total} [μkat l ⁻¹]	glucose [g l ⁻¹]	NaNO ₃ [g l ⁻¹]	K ₂ HPO ₄ [mg l ⁻¹]	gluconic acid [g l ⁻¹]	oxalic acid [g l ⁻¹]
0	-	-	-	-	80.0	3.0	1000	-	-
2	-	-	-	-	80.0	3.0	1000	-	-
5	-	-	-	-	77.0	3.0	1000	-	-
7.5	-	-	-	-	75.0	3.0	1000	-	-
10	0.24	0.16	0.22	0.38	73.0	3.0	1000	-	-
12.5	1.00	4.28	10.00	14.28	71.0	3.0	952	-	-
15	1.30	10.80	16.36	27.16	41.2	2.85	921	23.52	-
17.5	2.20	17.07	28.23	45.30	28.4	2.73	853	43.13	-
20	2.10	26.58	44.55	71.13	3.7	2.73	781	66.01	-
22.5	4.45	28.46	72.03	100.49	-	2.73	731	66.33	-
25	4.20	31.60	70.08	101.68	-	2.73	691	68.17	-
31	4.10	38.78	70.18	108.96	-	2.73	634	66.68	1.88
38	4.01	55.72	55.60	111.32	-	2.73	603	64.83	3.30
42	4.30	43.99	40.55	84.54	-	2.73	561	62.15	4.05
57	5.01	48.62	50.11	98.73	-	2.48	520	56.59	7.33
85	8.08	63.54	38.19	101.73	-	1.52	462	48.23	10.81
111	9.10	77.43	34.97	112.40	-	1.03	462	39.86	14.77
132	8.70	84.12	39.42	123.54	-	0.96	462	30.19	18.06
154	10.50	85.36	30.41	115.77	-	0.80	462	19.26	22.74

Table 9.1.1b: Batch cultivation of a recombinant *A. niger* in 5-L bioreactor using glucose as a sole C-source (growth in filamentous/micropellet)

Time [h]	CDW [g l ⁻¹]	GOx _{ext} [μkat l ⁻¹]	GOx _{int} [μkat l ⁻¹]	GOx _{total} [μkat l ⁻¹]	glucose [g l ⁻¹]	NaNO ₃ [g l ⁻¹]	K ₂ HPO ₄ [mg l ⁻¹]	gluconic acid [g l ⁻¹]	oxalic acid [g l ⁻¹]
0	-	-	-	-	80.0	3.0	1000	-	-
2	-	-	-	-	80.0	3.0	1000	-	-
5	-	-	-	-	77.0	3.0	1000	-	-
7.5	-	-	-	-	77.0	3.0	843	-	-
10	0.2	0.48	1.12	1.60	77.5	3.0	760	-	-
12.5	1.0	11.88	36.75	48.63	61.0	3.0	652	15.10	-
15	2.25	41.87	62.28	104.15	26.5	3.0	620	45.80	-
17.5	2.80	66.18	92.23	158.41	1.5	3.0	610	67.80	-
20	3.10	84.12	133.42	217.54	1.5	2.76	541	68.50	-
22.5	3.60	69.17	116.64	185.81	-	2.76	536	65.44	1.24
25	3.80	87.21	85.88	173.09	-	2.69	530	65.70	3.14
31	3.90	90.04	105.96	196.00	-	2.69	530	59.15	5.69
38	4.30	113.83	107.50	221.33	-	2.61	530	55.10	6.20
42	4.40	136.98	117.44	254.42	-	2.52	530	50.20	7.35
57	4.60	185.22	65.32	250.54	-	2.42	530	43.40	9.75
89	4.80	196.80	42.24	239.04	-	2.35	530	31.16	11.60
111	4.90	209.66	28.42	238.08	-	2.18	530	26.20	16.70
132	4.90	200.66	29.40	230.06	-	1.81	530	24.10	20.60
156	4.90	196.80	33.32	230.12	-	1.58	530	15.23	26.12
181	4.90	198.08	34.79	232.87	-	1.48	530	9.03	33.40

Table 9.1.2: Batch cultivation of a recombinant *A. niger* in 5-L bioreactor using fructose as carbon source

Time [h]	CDW [g l ⁻¹]	GOx _{ext} [μkat l ⁻¹]	GOx _{int} [μkat l ⁻¹]	GOx _{total} [μkat l ⁻¹]	fructose [g l ⁻¹]	NaNO ₃ [g l ⁻¹]	K ₂ HPO ₄ [mg l ⁻¹]	oxalic acid [g l ⁻¹]
0	-	-	-	-	80	3	1000	0
5	-	-	-	-	80	3	1000	0
10	1.41	-	-	-	77	3	1000	0
15	4.30	1.08	88.82	89.90	75.2	3	980	0
20	7.27	4.33	78.63	82.96	68.9	2.8	823	0
25	10.06	7.88	87.45	95.33	61.3	2.5	740	2.04
30	11.21	14.80	128.79	143.59	49.2	2.2	632	4.33
35	9.60	19.17	136.89	156.06	43.4	1.79	560	6.97
40	10.19	26.61	156.32	182.93	36.2	1.20	460	10.06
45	11.98	25.05	156.21	181.26	30.4	0.41	460	12.72
51	11.69	34.59	150.78	185.37	20.3	0.33	460	15.51
55	11.70	38.70	150.51	189.21	12.23	0.33	460	23.19
60	10.13	48.19	142.74	190.93	8.32	0.37	460	31.38
65	10.82	42.48	143.33	185.81	4.21	0.41	460	35.73
70	10.23	53.76	131.84	185.60	2.12	0.44	460	40.30
75	11.74	59.16	126.54	185.70	-	0.45	460	43.30
81	10.54	64.00	114.73	178.73	-	0.45	460	41.30
85	10.66	63.30	126.83	190.13	-	0.45	460	40.28
90	12.23	65.99	114.82	180.81	-	0.45	460	39.20
95	11.02	68.87	95.57	164.44	-	0.50	460	38.10
100	10.38	69.37	110.68	180.05	-	0.52	460	37.10
104	10.17	77.78	99.88	177.66	-	0.52	460	37.20
113	10.63	76.90	88.88	165.78	-	0.52	460	35.10
131	10.21	78.22	87.27	165.49	-	0.52	460	35.20

Table 9.1.3: Batch cultivation of a recombinant *A. niger* in 5-L bioreactor using xylose as C-source (800 rpm)

Time [h]	CDW [g l ⁻¹]	GOx _{ext} [μkat l ⁻¹]	GOx _{int} [μkat l ⁻¹]	GOx _{total} [μkat l ⁻¹]	xylose [g l ⁻¹]	NaNO ₃ [g l ⁻¹]	K ₂ HPO ₄ [mg l ⁻¹]	xylitol [g l ⁻¹]	oxalic acid [g l ⁻¹]
0	-	-	-	-	80.0	3.0	1000	-	-
5	-	-	-	-	79.20	3.0	1000	-	-
10	-	-	-	-	79.10	3.0	1000	-	-
15	1.40	7.45	29.11	36.56	76.64	3.0	1000	-	-
20	4.01	39.32	254.03	293.35	74.84	2.71	812	-	-
25	6.12	92.96	351.10	444.06	67.09	2.24	656	-	1.36
30	8.41	116.99	462.79	579.78	57.36	1.55	490	2.16	3.42
35	12.55	138.34	486.33	624.68	48.70	0.76	390	4.54	5.92
40	16.31	190.95	459.81	650.76	43.60	0.46	425	5.76	10.00
45	16.63	205.47	308.24	513.71	39.56	0.45	380	6.33	14.09
51	16.79	254.14	351.05	605.19	33.70	0.51	385	5.99	24.49
55	16.59	268.09	383.83	651.92	29.70	0.77	382	6.10	31.16
60	16.53	302.11	243.44	545.44	25.80	0.76	380	6.44	35.14
65	15.35	350.62	250.22	600.84	24.70	0.76	380	3.58	40.06
70	15.92	364.96	227.28	592.24	22.70	0.76	380	-	42.80
75	15.59	419.89	205.91	625.80	19.79	0.76	380	-	45.53
81	16.09	417.99	206.30	624.29	15.68	0.76	380	-	47.70
85	16.08	422.65	165.22	587.87	13.66	0.76	380	-	47.70
90	16.94	473.02	151.05	624.07	8.60	0.76	380	-	46.00
96	15.89	476.06	139.56	615.62	5.80	0.76	380	-	44.30
118	14.89	529.88	84.29	614.17	2.60	0.76	380	-	44.50

Table 9.1.4: Batch cultivation of a recombinant *A. niger* in 5-L bioreactor using xylose as C-source(500 rpm)

Time [h]	CDW [g l ⁻¹]	GOx _{ext} [μkat l ⁻¹]	GOx _{int} [μkat l ⁻¹]	GOx _{total} [μkat l ⁻¹]	xylose [g l ⁻¹]	NaNO ₃ [g l ⁻¹]	K ₂ HPO ₄ [mg l ⁻¹]	xylitol [g l ⁻¹]	oxalic acid [g l ⁻¹]
0	-	-	-	-	80.00	3.0	1000	-	-
5	-	-	-	-	80.00	3.0	998	-	-
10	-	-	-	-	78.86	3.0	939	-	-
15	3.29	-	-	-	73.24	3.0	822	-	-
17	4.45	6.26	93.80	100.60	ND	ND	ND	-	-
20	5.57	20.13	129.92	150.05	66.32	2.65	747	-	-
25	8.84	45.80	187.83	233.63	60.40	2.43	656	1.21	-
28	11.26	66.27	185.20	251.47	ND	ND	ND	ND	-
30	13.77	65.83	180.78	246.61	55.40	1.65	523	2.12	-
31	13.53	72.50	212.30	284.80	ND	ND	ND	ND	ND
34	14.78	85.48	242.50	327.98	ND	ND	ND	ND	ND
35	15.68	102.28	254.45	356.73	50.40	1.10	436	3.14	4.50
37	16.42	125.46	254.29	379.66	ND	ND	ND	ND	ND
40	18.17	128.23	253.14	381.37	44.20	0.52	365	4.17	5.632
45	21.02	162.20	242.21	404.41	36.60	0.45	296	5.21	7.624
51	21.50	186.05	212.10	398.15	24.56	0.50	320	4.13	9.42
55	22.40	268.11	219.34	487.45	18.07	0.62	365	3.12	11.93
60	23.15	334.95	196.84	531.79	14.76	0.62	341	2.11	12.94
65	24.06	398.82	253.19	652.01	10.15	0.60	293	1.21	15.45
70	25.95	436.03	225.17	661.20	6.87	0.60	283	-	16.15
75	27.30	472.28	240.92	713.20	3.68	0.60	277	-	19.48
81	28.42	481.75	260.85	742.60	1.35	0.60	273	-	21.32
85	24.60	506.63	291.48	798.11	-	0.60	268	-	23.34
90	21..80	551.48	231.00	782.48	-	0.60	302	-	24.24
96	21.90	582.01	233.57	815.58	-	0.60	305	-	26.05
118	21.77	679.33	184.75	864.08	-	0.60	305	-	24.30

ND: Not determined

Table 9.1.5: Batch cultivation of a recombinant *A. niger* in 5-L bioreactor using xylose as C-source (200 rpm)

Time [h]	CDW [g l ⁻¹]	GOx _{ext} [μkat l ⁻¹]	GOx _{int} [μkat l ⁻¹]	GOx _{total} [μkat l ⁻¹]	xylose [g l ⁻¹]	NaNO ₃ [g l ⁻¹]	K ₂ HPO ₄ [mg l ⁻¹]	xylitol [g l ⁻¹]	oxalic acid [g l ⁻¹]
0	-	-	-	-	80.0	3.0	1000	-	-
5	-	-	-	-	80.0	3.0	998	-	-
10	-	-	-	-	78.01	3.0	939	-	-
15	2.16	-	0.14	0.14	77.30	3.0	822	-	-
17	3.26	-	0.42	0.42	ND	ND	ND	-	-
20	4.00	0.10	6.10	6.20	75.30	2.65	747	-	-
23	5.11	2.47	25.28	27.75	ND	ND	ND	-	-
25	6.54	6.45	46.91	53.36	72.70	2.43	656	-	-
27	7.21	11.40	68.08	79.48	ND	ND	ND	-	-
30	8.43	17.30	83.67	100.97	66.40	1.65	523	-	-
35	11.77	34.47	140.52	174.99	55.50	1.10	436	1.2	-
37	12.12	46.05	160.82	206.87	ND	ND	ND	ND	ND
40	15.02	52.66	164.57	217.23	50.20	0.52	365	2.1	1.03
45	17.20	60.57	160.53	221.10	45.20	0.45	296	1.8	1.53
51	21.80	79.25	146.72	225.97	42.10	0.50	321	1.1	1.80
55	25.80	74.92	183.79	258.71	38.20	0.62	365	-	2.10
60	27.40	82.17	151.20	233.37	35.40	0.62	341	-	2.42
65	29.77	88.40	188.15	276.55	33.20	0.60	293	-	3.26
70	29.65	110.16	183.00	293.16	30.20	0.60	283	-	3.72
75	28.68	111.71	207.30	318.56	28.30	0.60	277	-	4.26
81	29.69	110.15	172.91	283.06	26.70	0.60	273	-	4.82
85	29.34	135.59	169.50	305.09	24.70	0.60	269	-	4.70
90	30.56	145.52	166.90	312.42	23.20	0.60	302	-	5.31
96	30.40	155.69	152.77	308.46	22.10	0.60	305	-	5.93
118	29.60	160.44	110.85	217.29	21.40	0.60	305	-	6.26

ND: Not determined

Table 9.1.6: Batch cultivation of a recombinant *A. niger* in 5-L bioreactor using a mixed substrate culture (xylose/xylan), 800 rpm.

Time [h]	CDW [g l ⁻¹]	GOx _{ext} [μkat l ⁻¹]	GOx _{int} [μkat l ⁻¹]	GOx _{total} [μkat l ⁻¹]	xylose [g l ⁻¹]	NaNO ₃ [g l ⁻¹]	K ₂ HPO ₄ [mg l ⁻¹]	xylitol [g l ⁻¹]	oxalic acid [g l ⁻¹]
0	-	-	-	-	60.00	3.00	1000	-	-
5	-	-	-	-	57.70	3.00	981	-	-
10	1.54	-	-	-	53.99	2.85	981	-	-
15	3.72	0.32	6.50	6.82	52.28	2.65	965	-	-
20	6.76	24.23	269.08	293.31	45.30	2.17	884	-	-
25	9.92	84.04	319.65	403.69	40.20	1.70	739	-	-
30	14.76	107.62	388.563	496.18	34.20	0.13	674	-	-
35	15.73	151.51	389.48	540.99	24.83	0.14	318	2.61	2.54
39	16.08	168.70	423.30	592.00	20.81	0.14	399	3.18	3.62
42	15.89	185.09	414.00	599.09	17.69	0.19	431	3.90	4.92
47	16.48	263.37	370.42	633.79	16.36	0.19	443	4.24	6.81
52	16.58	307.45	336.93	644.38	12.34	0.19	463	7.86	8.40
56	16.88	335.85	323.90	659.75	10.57	0.19	463	6.48	10.30
62	16.97	382.60	311.68	694.28	9.24	0.20	496	4.82	11.39
69	17.25	445.57	315.59	761.16	6.58	0.20	593	4.76	12.97
75	17.11	499.95	284.26	784.21	5.22	0.28	544	4.31	14.69
83	16.95	521.80	295.33	817.13	2.29	0.31	544	3.33	17.11
87	16.80	534.31	312.73	847.04	-	0.31	544	3.58	17.91
95	17.00	539.56	298.34	837.90	-	0.31	544	3.53	20.17
108	17.03	552.43	245.24	797.67	-	0.31	544	2.99	21.95
114	16.30	548.44	202.10	750.54	-	0.31	544	2.83	23.89

Lebenslauf

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